ASSEMBLY OF CENTROMERIC CHROMATIN IN FISSION YEAST

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PREFACE

This thesis was composed by myself and the research presented is my own unless otherwise stated.

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ABSTRACT

Genome stability is dependent on the accurate segregation of chromosomes during cell division. The centromere is the specialised chromosomal locus that ensures the normal transmission of chromosomes during mitosis and meiosis. A defining feature of centromeres is the presence of the histone H3 variant CENP-A, which is a strong candidate for the epigenetic mark that specifies centromere identity. The mechanism by which CENP-A is delivered to and incorporated exclusively at chromatin at centromeres is, as yet, not fully understood.

The three fission yeast centromeres resemble centromeres of higher eukaryotes in both function and structure. Fission yeast centromeres are composed of two distinct domains: a central domain where the kinetochore assembles, which is surrounded on both sides by outer repeat heterochromatin. Marker genes inserted within the central domain or the outer repeats are transcriptionally silenced. Mutants that alleviate silencing at the central domain or the outer repeat domain have been shown to be defective in kinetochore and heterochromatin integrity, respectively. A screen performed to identify mutants that specifically alleviate silencing at the central domain, identified the *sim* (silencing in the middle of the centromere) mutants. The *sim6* mutant was unusual in that it was found to alleviate both central domain and outer repeat silencing and suggests that there may be cross talk between kinetochore assembly and the integrity of neighbouring heterochromatin. To identify other factors with a similar phenotype a screen was performed in which the *cos* (central core and outer repeat silencing) mutants were isolated. Several mutants allelic to *sim6*$^+$ (renamed *cos1*$^+$) were isolated. *cos1*$^+$ is allelic to fission yeast *mcl1*$^+$ (mini-chromosome loss 1) and may function to ensure that features of silent chromatin and sister chromatin cohesion are properly maintained after replication.

sim3 mutants were found to specifically alleviate silencing at the central domain. Sim3 is homologous to the histone binding proteins mammalian NASP (nuclear autoantigenic sperm protein) and *Xenopus laevis* N1/N2. Cells with defective Sim3 have reduced levels CENP-A^{Cnp1} at centromeres and subsequently display defects in mitotic chromosome segregation. Sim3 interacts directly with CENP-A^{Cnp1} and is a highly abundant protein that is not concentrated at centromeres, but rather is distributed throughout the entire nucleoplasm. In higher eukaryotes, CENP-A is deposited at centromeres in a manner that is independent of DNA replication. In fission yeast, Sim3 is required for the deposition of newly synthesized CENP-A^{Cnp1} at centromeres in the G2 stage of the cell cycle. Sim3 may be acting as a CENP-A^{Cnp1} 'escort', assisting in the replication independent assembly of CENP-A^{Cnp1} chromatin at centromeres, however Sim3 may also contribute to the loading of Sim3 at other stages of the cell cycle. It remains to be determined whether NASP/N1/N2 plays a similar role in metazoa.

ABBREVIATIONS

ATP	adenosine tri phosphate
bp	base pair
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
cdc	cell division cycle
cDNA	complementary deoxyribonucleic acid
ChIP	chromatin immunoprecipitation
CENP	centromere protein
C-terminal	carboxy-terminal
DAPI	4,6-diamino-2-phenylindole
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxy-nucleotide triphosphate
dsRNA	double stranded ribonucleic acid
ECL	enhaced chemiluminescence
EDTA	ethylene di-amine tetra acetic acid
EM	electron microscopy
EMM	edinburgh minimal media
FITC	fluorescein isothiocyanate
FISH	fluorescent *in situ* hybridisation
5-FOA	5-fluoro-orotic acid
GFP	green fluorescent protein
H3K9me2	histone H3 dimethylated on lysine 9
H3K4me2	histone H3 dimethylated on lysine 4
HA	haemagglutinin
HDAC	histone deacetylase
HP1	heterochromatin protein 1
IgG	immunoglobulin
IP	immunoprecipitation

kb	kilobase
kDa	kilodalton
LB	Luria-Bertani broth
Mbp	mega basepair
ME	malt extract
MNase	micrococcal nuclease
MT	microtubule
nmt	no message in thiamine
N-terminal	amino-terminal
mRNA	messenger ribonucleic acid
ORF	open reading frame
PAGE	poly-acrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEV	position effect variegation
P*inv*	invertase promoter
Pol	polymerase
PMG	*pombe* media glutamate
RDRC	RNA-Directed RNA polymerase Complex
RITS	RNA-mediated Initiation of Transcriptional Silencing
RNA	ribonucleic acid
RNAi	RNA interference
rpm	rotation per minute
RT	room temperature
SDS	sodium dodecyl sulphate
siRNA	small interfering ribonucleic acid
TBE	Tris-Borate EDTA
TBZ	thiobendazole
TRITC	tetra-methyl rhodamine isothiocyanate
tRNA	transfer RNA

ts	temperature sensitive
TSA	trichostatin A
Tween	polyoxyethylenesorbitan monolaurate
YES	Yeast Extract Supplemented

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CHAPTER 1

INTRODUCTION

The genetic information in the eukaryotic cell is organised into chromatin. Nucleosomes are the fundamental units of chromatin and consist of 146 bp of DNA wrapped around an octamer of two of each of the highly conserved histones H2A, H2B, H3 and H4 (Luger et al., 1997, reviewed in Wolffe and Kurumizaka, 1998). Nucleosomes are then organised into a higher ordered structure called a 10 nm fibre, which is then compacted further into a 30 nm chromatin fibre, which facilitates the packing of DNA into the nucleus (reviewed by Robinson and Rhodes, 2006). During cell division chromosomal DNA is condensed into distinct entities to facilitate the segregation of sister chromatids into daughter nuclei. The maintenance of genome integrity is crucially dependent on accurate segregation of chromosomes during mitosis and meiosis. The mechanism of chromosome segregation is conserved in all eukaryotes: DNA content in the cell is doubled at replication and must be divided evenly during cell division. Exactly how the cell manages to complete this complex feat is not fully understood. Mistakes that result in gain or loss of chromosomes (aneuploidy) are associated with disease that may lead to death in humans (reviewed by Cahill et al., 1998). Aneuploidy in human meiotic cells is known to generate severe congenital disorders such as Down's syndrome (reviewed by Nicolaidis and Peterson, 1998). For example, chromosome instability and aneuploidy in somatic mitotic cells may predispose cells to tumour development and accelerate tumour progression (reviewed by Sen, 2000).

THE CENTROMERE AND KINETOCHORE

The centromere is the highly specialised chromosomal locus that ensures the delivery of one copy of each chromosome to each daughter cell at cell division. The centromere can be defined cytologically as the primary constriction visible on condensed chromosomes. The kinetochore is the protein complex assembled at each centromere and serves as the attachment site for spindle microtubules and is the site at which motors generate forces to power chromosome movement and segregation at cell division (Pluta et al., 1995). To accurately pass on genetic information to daughter cells, newly replicated sister chromatids must attach bilaterally to the microtubules that emanate from opposite poles and the centromere and kinetochore proteins play a critical role in this essential process.

In this chapter, I will review what is known about centromere structure and function and discuss the evidence supporting the fact that eukaryotic centromeres are regulated in an epigenetic manner. I will describe the organisation of the fission yeast (*Schizosaccharomyces*

pombe) centromere in detail and discuss the proteins that associate with centromeres, including the histone H3 variant CENP-A. This chapter also includes a review of the role of histone variants in the cell and of the mechanisms currently proposed to facilitate the assembly of CENP-A specifically at centromeres.

EPIGENETIC REGULATION OF CENTROMERES

Surprisingly, despite its important function, the site of centromere formation is not governed by DNA sequence and centromeric DNA sequences are neither necessary nor sufficient to mark the site of centromere formation and function. Thus, it is likely that the specification of centromere identity is regulated in an epigenetic manner (Figure 1-1). Dicentric chromosomes, either engineered or found to occur naturally on abnormal chromosomes, contain two regions of DNA that are capable of functioning as centromeres (Sullivan and Willard, 1998, Higgins et al., 1999). One such abnormal chromosome in humans results from a Robertsonian translocation, where the two short arms from different chromosomes are linked together to form a dicentric chromosome (Warburton et al., 1997). The presence of two potential centromeres could lead to catastrophic events during cell division, such as the formation of anaphase bridges that result in genome instability. However, dicentric chromosomes in flies and humans can be stably transmitted, due to an inactivation event where only one centromere is active (Sullivan and Willard, 1998, Warburton et al., 1997, Sullivan and Schwartz, 1995, Agudo et al., 2000). In dicentric chromosomes, the kinetochore proteins are only visualised at the active centromere and not at the inactive centromere (Warburton et al., 1997, Earnshaw et al., 1989, Sullivan and Schwartz, 1995). In *Drosophila*, both centromeres of a dicentric chromosome have been shown to be active, but only one at a time, suggesting the activation and inactivation of a given centromere is a plastic process (Agudo et al., 2000). Exactly how one centromere is selected to be active and the other to remain dormant is not fully understood.

It is also clear that the presence of centromeric DNA is not always necessary for the propagation of a functional centromere at a particular locus. Neocentromeres have been identified in humans and flies, where the kinetochore is found to assemble at non-centromeric DNA (Depinet et al., 1997, Du Sart et al., 1997, Williams et al., 1998, Lo et al., 2001, Saffery et al., 2000, Maggert and Karpen, 2001, Warburton et al., 1997). The best characterised is the human neocentromere spanning 400 kb at position 10q25.2 on the marker chromosome mardel (10), which is formed following a complex rearrangement of normal chromosome 10 (Du Sart et al., 1997). Sequencing of 80 kb of this region, which is capable of acting as a centromere, reveals no difference in this DNA sequence and the sequence of the corresponding normal region from the patient's father (Barry et al., 1999, 2000). Moreover, human neocentromeres are indistinguishable from their alpha satellite-containing normal counterparts in terms of protein composition and distribution, with the

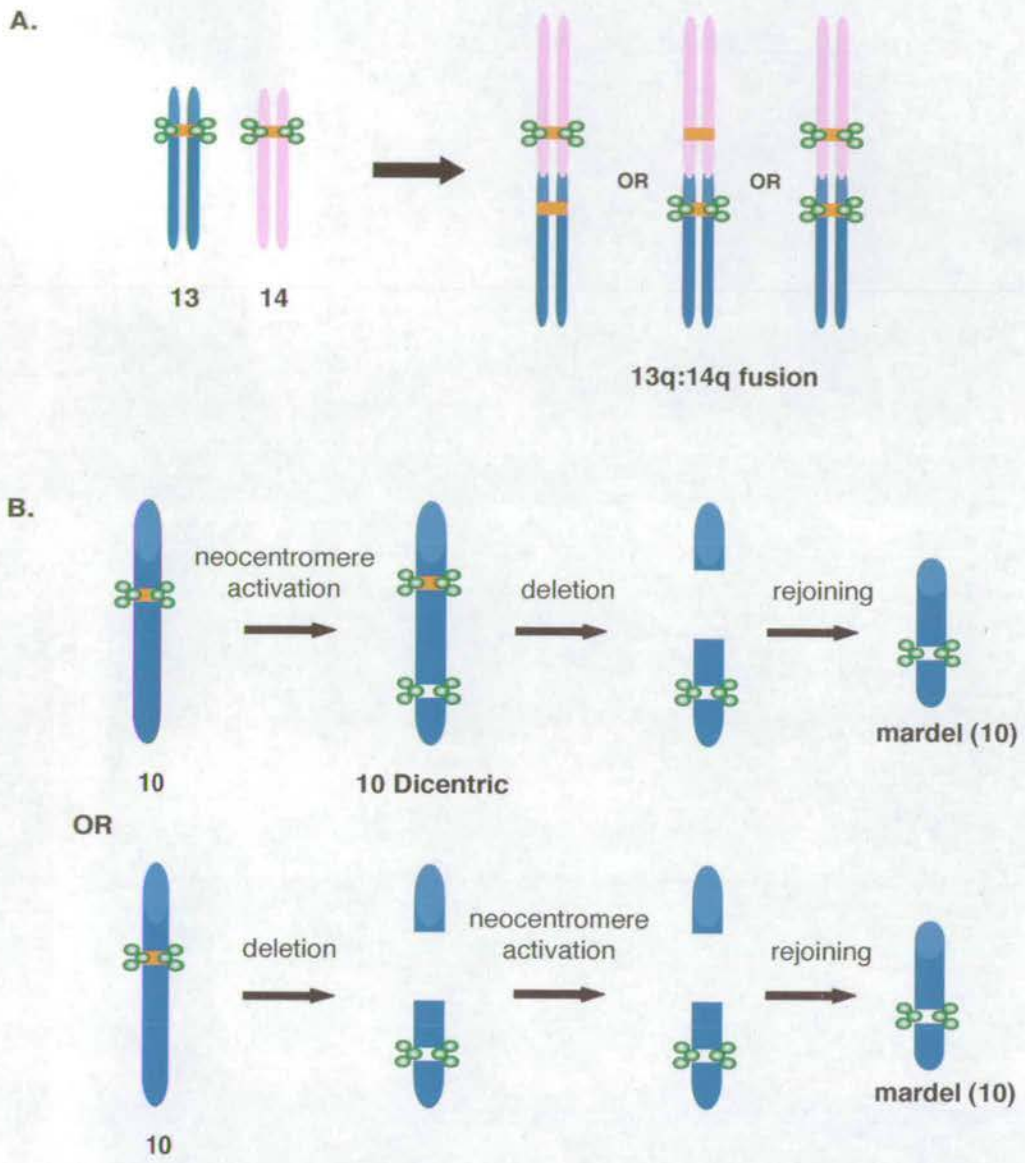


Figure 1-1. Centromeres are regulated epigenetically (adapted from Karpen and Allshire, 1997).

A. Normal centromere-associated DNA alone does not ensure centromere function. Fusion of two human chromosomes such as chromosome 13 and 14, leads to the formation of a dicentric chromosome where one or the other centromere can be inactivated. Functional kinetochore components (green spheres) can be associated with the 13 or 14 α-satellite containing regions (red bar), suggesting that activation/inactivation of a given centromere might alternate within a population.

B. Active centromeres can be formed at sites that do not contain sequences ordinarily associated with a centromere. In one well-studied human neocentromere (mardel(10)), kinetochore proteins (green spheres) reside at site 10q25 (white bar) that lacks α-satellite repeats (red bar). Mardel(10) might have arisen from activation of 10q25 on a normal chromosome, followed by breakage and rejoining (top). Alternatively, deletion might have preceeded neocentromere activation (bottom).

exception of the centromere sequence specific binding protein CENP-B that is absent from neocentromeres (Saffery et al., 2000). Neocentromeres have also been described in *Drosophila melanogaster* (Murphy and Karpen, 1995, Williams et al., 1998). Acentric derivatives of the *Drosophila Dp1187* mini-chromosome, that lack repetitive sequences associated with the normal *Dp1187* centromere, are stably transmitted through mitosis and meiosis (Murphy and Karpen, 1995). Thus, it appears that the centromere-deleted mini-chromosome can acquire neocentromere activity. Experiments have shown that the formation of experimentally induced neocentromeres in *Drosophila* requires proximity to a functional centromere, however the mechanism for this 'spreading' *in cis* of centromere activity is not known (Maggert and Karpen, 2001).

Evidence from fission yeast also demonstrates that centromere formation and kinetochore assembly are regulated epigenetically. Truncated centromere constructs, when transformed into *S. pombe*, are metastable and can adopt two different states: in most transformants the construct is highly unstable and centromere activity is absent; however in a low frequency of transformants (0.6% of cells) the construct is stably propagated, owing to the assembly of an active centromere (Steiner and Clarke, 1994). This suggests that centromeric DNA can be associated with two functionally different states and moreover, once established the active state is perpetuated for many cell divisions (Steiner and Clarke, 1994). A second example of the epigenetic regulation of centromeres in fission yeast comes from an experiment where *S. pombe* cells are treated with drugs that induce histone hyperacetylation. Following drug treatment, defects in chromosome function persist in daughter cells for a number of generations, even after removal of the drug (Ekwall et al., 1997). In addition, some cells switch back to normal levels of histone acetylation, albeit at a low frequency, and proper centromere function is restored. Recent evidence also demonstrates that functional centromeric chromatin is specified epigenetically in the yeast *Candida albicans* (Baum et al., 2006). In this study, a mini-chromosome that is stably maintained in mitotic divisions, cannot be stably propagated when introduced back into *C. albicans* cells by transformation as naked DNA, suggesting that the pre-existing chromatin state is entirely responsible for the propagation of functional centromeric chromatin (Baum et al., 2006).

The fact that centromeres can be inactivated, as occurs in the case of dicentric chromosomes, the ability of neocentromeres to form at non-centromeric sites and evidence from fission yeast and *C. albicans*, together suggest that there is a certain degree of plasticity involved in the assembly of the kinetochore and the activation of a centromere. These observations strengthen the proposal that normal centromere function is regulated in an epigenetic manner, which can account for both the stability and plasticity of the centromere.

DEFINING CENTROMERE IDENTITY

A remarkable feature of centromeres is that, although their general organisation, function and the majority of kinetochore proteins that assemble at centromeres are conserved across species, the DNA sequences found at centromeres are not (reviewed by Sullivan et al., 2001). However, in general, centromeres do exhibit two defining features across evolution: the presence of stretches of tandemly repeated DNA sequences and the presence of a specialised centromeric chromatin containing the histone H3 variant CENP-A (centromere protein A). CENP-A is localised cytologically at the inner plate of the centromere (Warburton et al., 1997) and is proposed to be the epigenetic mark that specifies the identity of an active centromere. Furthermore, CENP-A is important for initiating the assembly of most other kinetochore components, which are delocalised in the absence of functional CENP-A (Stoler et al., 1995, Buchwitz et al., 1999, Howman et al., 2000, Takahashi et al., 2000, Blower and Karpen, 2001). CENP-A is called Cnp1 in fission yeast (Takahashi et al., 2000), Cse4p in budding yeast (Meluh et al., 1998) and CID in *Drosophila* (Henikoff et al., 2000). Repetitive sequences at eukaryotic centromeres other than budding yeast, vary from approximately 40 to 100kb in fission yeast, to several mega bases in humans as described below (see Figure 1-2). A comparison of the molecular organisation of the vertebrate, fission yeast and budding yeast centromere/kinetochore is illustrated in Figure 1-3.

Saccharomyces cerevisiae centromeres

The centromeric DNA of the budding yeast *Saccharomyces cerevisiae* is a 125 bp sequence that is divided into three distinct elements, designated CDEI, CDEII and CDEIII which are common to all 16 chromosomes (reviewed by Cheeseman et al., 2002). CDEII is an 80 bp AT rich sequence where the single Cse4p-containing (*S. cerevisiae* CENP-A^{Cse4p}) nucleosome of the budding yeast 'point centromere' is assembled, and is required for faithful chromosome transmission (Stoler et al., 1995, Keith and Fitzgerald-Hayes, 2000). The gene encoding *CSE4* is essential and cells bearing mutations in *cse4* arrest in G2/M phase of the cell cycle (Stoler et al., 1995, Keith and Fitzgerald-Hayes, 2000). More recently, it has been shown that depletion of Cse4p fused to an N degron sequence results in the delocalisation of many kinetochore proteins by chromatin IP (Collins et al., 2005). CDEIII is essential for centromere function and serves as a binding site for the CBF3 complex consisting of Ndc10p, Cep3p, Ctf13p and Skp1p, which is essential for kinetochore function (Lechner and Carbon, 1991, Goh and Kilmartin, 1993, Sorger et al., 1995). CDEI is non-essential for viability and serves as a binding site for the non-essential kinetochore protein Cbf1p (centromere binding factor 1) (Mellor et al., 1990). The central kinetochore complex Ctf19 (Ctf19p, Mcm21p and Okp1p) is found at the interface with centromeric DNA (Oritz et al., 1999) and links together the Ctf3 complex (Ctf3p, Mcm16p and Mcm22p) (Measday et al., 2002) and the essential Ndc80 complex (Ndc80p, Spc24p, Spc25p and Nuf2p) (Janke et al.,

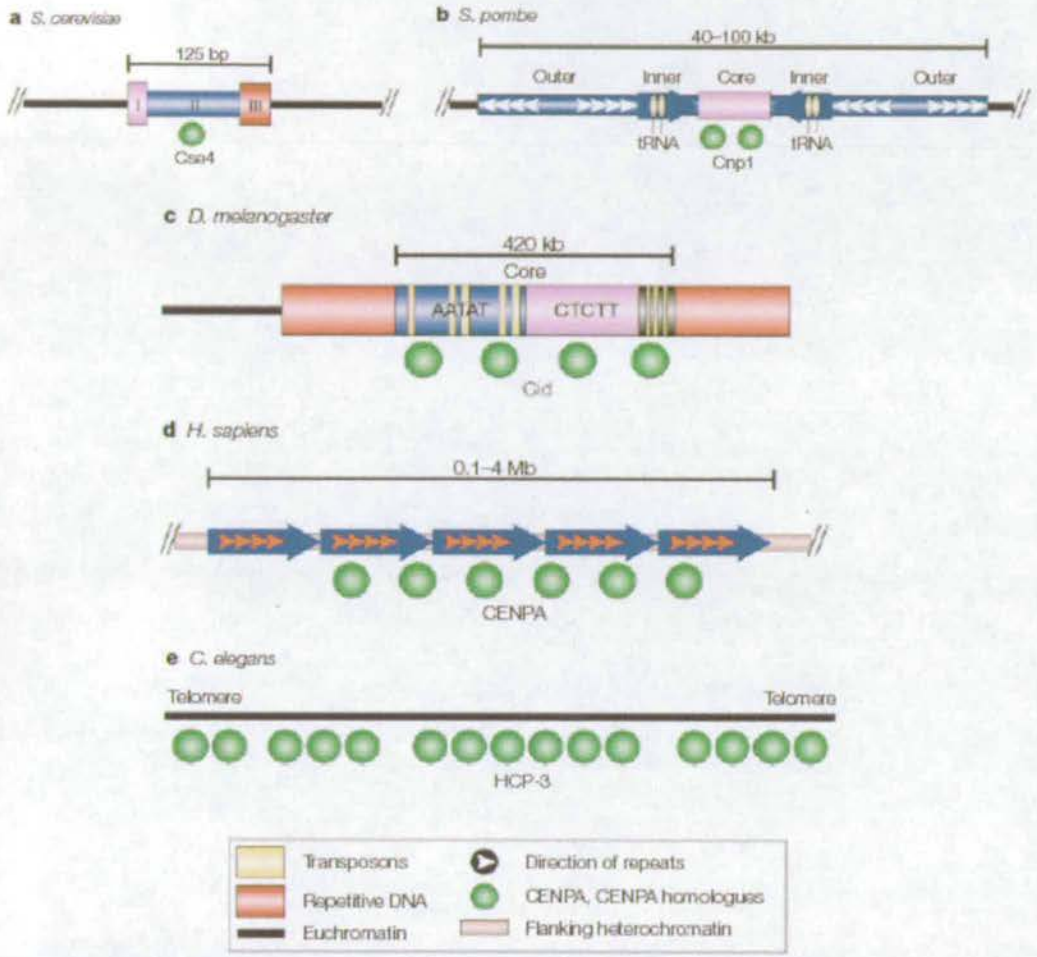


Figure 1-2. Centromeric DNA and conservation of CENP-A at structurally distinct centromeres (from Sullivan et al., 2001).

CENP-A and its homologues are shown in green. **a)** Budding yeast *Saccharomyces cerevisiae* 'point' centromere function depends on three DNA elements designated CDEI, CDEII and CDEIII. CENP-A^{Cse4p} localises to CDEII. **b)** Fission yeast *Schizosaccharomyces pombe* centromeres comprise a unique central core domain, where CENP-A^{Cnp1} assembles, and is flanked either side by repetitive inner and outer repeat sequences. tRNA genes act as boundary elements. **c)** *Drosophila melanogaster* centromeres, from the *Dp1187* mini-chromosome, consist of AATAT and AAGAG satellite repeats (AAGAG = CTCTT in diagram) interspersed by transposons and flanked by other repetitive DNA (red). CENP-A^{CID} assembles over the 420 kb region. **d)** Human centromeres contain large arrays of tandemly repeated 171 bp α-satellite DNA (red), arranged into higher order repeats (blue) that span several megabases in length. CENP-A assembles over a portion of the α-satellite repeats only. **e)** *Caenorhabditis elegans* holocentric centromeres assemble CENP-A^{HCP-3} and kinetochores along the length of the entire chromosome.

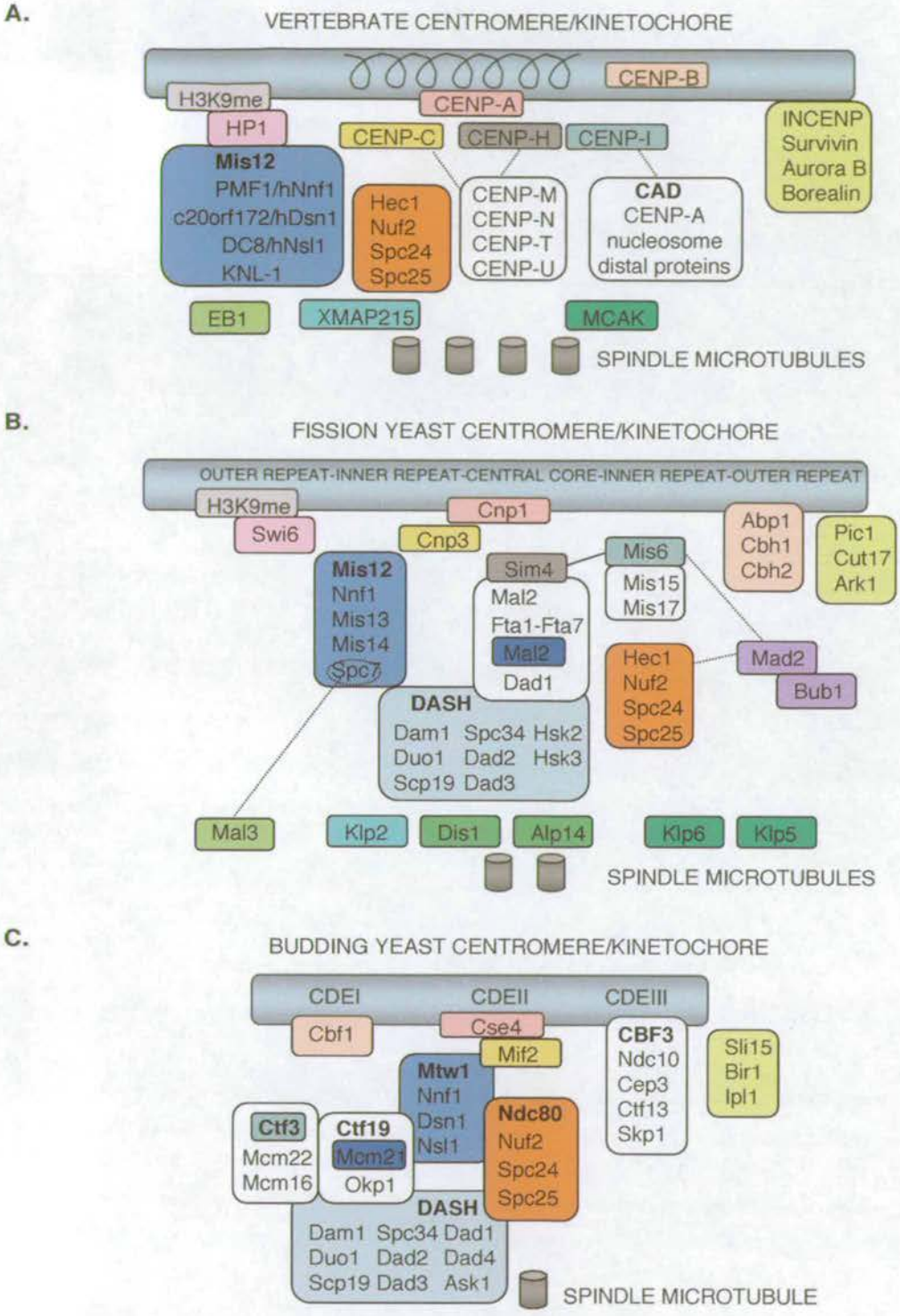


Figure 1-3. Comparison of molecular organisation of kinetochore proteins between vertebrates and fungi (adapted from review by Chan et al. (2005)).

Schematic representation of the spatial distribution of: **A**. the vertebrate kinetochore, **B**. the fission yeast kinetochore, **C**. the budding yeast kinetochore. Proteins that physically interact with each other are depicted as shapes that contact each other or are linked by dotted lines. All protein complexes are depicted with their subunits as a single shape. Conserved proteins/complexes among organisms are indicated by colour. White shapes indicate proteins or complexes where no clear homologues have been described.

2001, Wigge and Kilmartin, 2001). The Dam1p complex is found at the outer kinetochore and interacts physically with both the Ctf3p and Ndc80p complexes (Cheeseman et al., 2001, Measday et al., 2002). The Dam1p complex consists of microtubule associated proteins (MAPs) and mediates kinetochore attachments to the spindle in conjunction with other MAPs (reviewed by Cheeseman et al., 2002).

The chromatin organisation of *S. cerevisiae* centromeres is distinct as the core CEN sequence (CDEI + CDEII + CDEIII) of each centromere is flanked on either side by DNase I hypersensitive sites and an array of positioned nucleosomes (Bloom and Carbon, 1982). In addition, a cluster of three closely spaced *Dra*I restriction endonuclease sites at the CDEII region of *CEN3* are protected from digestion in the presence of a functional centromere/kinetochore (Saunders et al., 1990). Kinetochore mutants, such as those with defective *cse4* show increased accessibility of the *Dra*I clusters, indicating that the proper integrity of centromeric nucleosomes is required for normal centromere function (Saunders et al., 1988, Meluh et al., 1998).

Drosophila melanogaster centromeres

In the fruit fly *Drosophila melanogaster*, analysis of the *Dp1187* mini-chromosomes has defined a 420 kb region that is both necessary and sufficient for centromere activity (Murphy and Karpen, 1995, Sun et al., 1997). This sequence is composed of AATAT and AAGAG satellites, which make up about 85% of the total 420 kb and is interspersed with transposable elements, which comprise about 10% of the total 420 kb, along with an A-T rich region at the right end of the centromere (Sun et al., 1997). The AATAT and AAGAG satellites, however, are distributed throughout the genome, predominantly at regions that never act as functional centromeres, and are alone unlikely to specify the site of centromere activity (Sun et al., 1997). As the AAGAG satellite block is essential for full centromere function, it has been proposed to include the sites for kinetochore assembly (Murphy and Karpen, 1995). In contrast, mini-chromosome derivatives with deletions of the AATAT satellite/transposon region (designated *Bora Bora*) displayed diminished stability and these sequences may play a more auxiliary role in centromere function, such as the maintenance of sister chromatin cohesion (Murphy and Karpen, 1995). This suggests that *Drosophila* centromeres may be composed of two domains and is supported by cytological studies showing that the domains where the kinetochore proteins localise are flanked by, but do not overlap with, the neighbouring centric heterochromatin domains where proteins such as the heterochromatin marker Su(var)2-5 (heterochromatin protein 1 equivalent, HP1) localise (reviewed by Sullivan et al., 2001).

Cytological studies have shown that *Drosophila* CENP-A, known as CID (centromere identifier), is localised to the inner kinetochore and proper CENP-A^{CID} localisation

correlates with centromere function (Blower and Karpen, 2001). In addition CENP-A^{CID} is required for cell cycle progression, accurate chromosome segregation in mitosis and kinetochore assembly in flies (Blower and Karpen, 2001). Examination of the linear structure of stretched chromosomes showed that fly centromeric chromatin is composed of interspersed blocks of CENP-A^{CID} and histone H3 nucleosomes and a similar pattern was observed in stretched human chromosome fibres (Blower et al., 2002). These discontinuous blocks of CENP-A^{CID} and H3 containing chromatin are organised into distinct domains on three-dimensional metaphase chromosomes, which may have distinct functional properties (Blower et al., 2002).

Caenorhabditis elegans holocentric centromeres

Holocentric eukaryotes, such as the nematode *C. elegans*, are unusual in that they have diffuse kinetochores that form along the entire length of their chromosomes and attach to spindle microtubules at many points along their length (reviewed by Pimpinelli and Goday, 1989). Although the specific DNA sequences that define the site of kinetochore assembly have not been defined, the *C. elegans* CENP-A homologue HCP-3 has been shown to localise along the entire length of the mitotic chromosome (Buchwitz et al., 1999) and is essential for kinetochore assembly and mitotic chromosome segregation (Oegema et al., 2001, Desai et al., 2003). Remarkably, CENP-A^{HCP-3} is dispensable for chromosome segregation in meiosis in *C. elegans* and holocentric chromosomes appear to be able to recruit kinetochore proteins to the centromere via a mechanism that is independent of CENP-A^{HCP-3} (Monen et al., 2005).

Mammalian centromeres

Mammalian centromeres are larger and much more complex than those of yeast or the fruit fly. Human centromeres are composed of a chromosome specific, higher-order arrangement of a tandemly repeated, A-T rich 171 bp unit termed α-satellite (Willard et al., 1985). These higher-order arrays (also called alphoid DNA) span 0.3 to 5 Mbps of DNA, encompassing the same α-satellite element repeated hundreds or thousands of times within a given centromere locus (Willard et al., 1989). Insertion of this alphoid DNA at ectopic, non-centromeric sites does not result in the formation of a functional kinetochore (Haaf et al., 1992, Warburton and Cooke, 1997). Furthermore, the inactivation of one of the centromeres of a dicentric chromosome (Sullivan and Willard, 1998, Sullivan and Schwartz, 1995) demonstrates that while alphoid DNA is the preferred site for centromere formation, it is not absolutely necessary for centromere formation. CENP-A nucleosomes associate with α-satellite DNA at human centromeres (Vafa and Sullivan, 1997). It is estimated, however, that CENP-A localises to only one half to two thirds of the entire centromere-specific α-satellite array (Warburton et al., 1997, Blower et al., 2002, Lam et al., 2006). Moreover, analysis of stretched human chromatin fibres revealed that CENP-A- and H3-containing nucleosomes are interspersed within the centromere (Blower et al., 2002). These observations have suggested a model where the CENP-A and H3 chromatin at centromeres

coalesce to form a unique three-dimensional structure, which may play a role in distinguishing the centromere from the rest of the chromosome (Blower et al., 2002, Schueler and Sullivan, 2006; see Figure 1-8).

Many human α-satellite repeats contain a 17 bp conserved binding site for the sequence specific DNA binding protein known as centromere protein B (CENP-B) (Masumoto et al., 1989), suggesting that proteins may be recruited to the centromere in a DNA sequence dependent manner. CENP-B contains a dimerisation domain and binding to the CENP-B box brings two CENP-B boxes into close proximity (Kitagawa et al., 1995), which is thought to contribute to the higher order packing of chromatin at the centromere. Transformation of α-satellite containing CENP-B boxes into cultured human HT1080 cells has been shown to allow *de novo* kinetochore assembly (Ohzeki et al., 2002). This suggests that CENP-B boxes, and thus maybe CENP-B may be required for the establishment of the kinetochore on α-satellite DNA. However, mice that lack CENP-B are viable and do not display any significant defects in chromosome segregation (Hudson et al., 1998, Kapoor et al., 1998). In addition, neocentromeres such as mardel (10) form at chromosomal regions completely devoid of α-satellite repeats and lack CENP-B, but are stably inherited through many generations of cell division (Voullaire et al., 1993, Barry et al., 1999).

Primate centromeres are also composed of two major types of α-satellite (He et al., 1998): a highly regular α-I satellite where CENP-A is bound (Ando et al., 2002) and a more varied α-II satellite where cohesins are concentrated. Mouse (*Mus musculus*) centromeres are composed of two classes of repetitive DNA: major satellites comprising tandem arrays of a 234 bp unit and minor satellites comprising a 120 bp unit organised into uninterrupted blocks of tandem arrays of approximately 2,500 copies per chromosome, where the kinetochore proteins associate (reviewed by Mitchell, 1996). *Arabidopsis thaliana* centromeres are composed of a 178 bp α-satellite repetitive array (Hall et al., 2003) that is different from human α-satellite, and it is here that the centromeric H3 variant CENP-A^{HTR12} assembles (Talbert et al., 2002).

FISSION YEAST CENTROMERES

Centromere organisation

The fission yeast *Schizosaccharomyces pombe* has three chromosomes with centromeres that vary from 35 kb to over 100 kb in length (Figure 1-4). Fission yeast centromere I (*cen1*) occupies 35 kb and is on the largest chromosome, *cen2* is 65 kb and *cen3* is 110 kb and is on the smallest chromosome (Takahashi et al., 1992, Steiner et al., 1993, Wood et al., 2002).

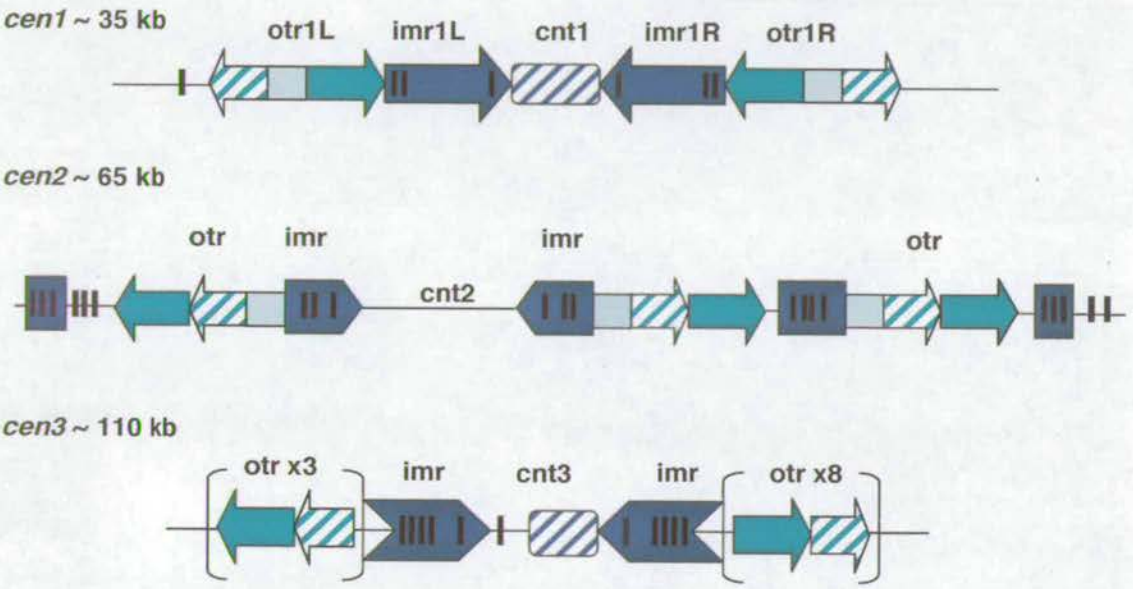


Figure 1-4. Organisation of the three fission yeast centromeres (adapted from Takahashi et al., 1992).

The three fission yeast centromeres share the same overall structure, in which a central core (*cnt*) is surrounded by innermost repeats (*imr*) and outer repeats (*otr*). The arrows in the outer repeats (*otr*) represent the repetitive elements *dg* (filled arrows) and *dh* (hatched arrows). The large purple arrows represent the innermost repeats (*imr*) and are largely non homologous among the three centromeres. The central core sequence (*cnt*) is non homologous, except for regions in *cen1* and *cen3* represented by purple hatched boxes. The vertical lines indicate the tRNA genes. *cnt* and *imr* comprise the central domain where CENP-A^{Cnp1} and the kinetochore assembles; *otr* repeats make up the heterochromatin domain.

Each centromere consists of a central core (*cnt*) sequence of 4 – 7 kb in length, which is non-repetitive and AT rich. *cen1* and *cen3* share a 3.3 kb element, called 'TM' which is 99% identical in sequence between the two centromeres. *cen2* has a 1.5 kb element that is 48% identical to TM (Wood et al., 2002). Each *cnt* is surrounded by inner-most repeat (*imr*) sequences that are unique to each centromere. Together the *cnt* and *imr* comprise the central domain of the fission yeast centromere. The central domain is flanked either side by outer repeat regions composed of *'dg'* and *'dh'* elements (also known as *K* and *L* repeats). The outer repeat elements are highly homologous among the three centromeres; the *dg* repeats show 97% identity and *dh* repeats show 48% identity. The number of repeats and total size of the outer region is variable among the three centromeres: 2 repeats or 20 kb for *cen1*, 3 repeats or approximately 60 kb for *cen2* and 13 repeats or greater than 100 kb for *cen3*. Clusters of tRNA genes are found within the *imr* repeats of all three centromeres and within the *cnt* of *cen3* (Kuhn et al., 1991, Takahashi et al., 1991). The tRNA clusters at the *imr* repeats of *cen1* have been shown to act as barriers and mark the transition between the central and outer repeat domains (Partridge et al., 2000, Scott et al., 2006). tRNA genes are also found outside the outer repeat regions on the right and left extremities, except for the right arm of *cen1*, however the function of these tRNA clusters is unknown, although it is possible that they may act as boundaries that define the outer limits of centromeric heterochromatin.

The central domain (*cnt* and *imr*) has a unique chromatin structure and produces a non-nucleosomal 'smear' pattern on digestion with micrococcal nuclease (MNase) (Polizzi and Clarke, 1991, Takahashi et al., 1992). In contrast, the chromatin at the outer repeats is regular and gives a normal nucleosomal ladder pattern on MNase digestion (Polizzi and Clarke, 1991). Numerous studies have dissected the DNA element required for centromere function (Niwa et al., 1989, Hahnenberger et al., 1989, 1991, Masumoto et al., 1990, Clarke and Baum, 1990, Baum et al., 1994). The minimal requirement sufficient to establish a functional *S. pombe* centromere is a portion of the central core (*cnt*) with *imr* repeats and at least one K type (*dg*) outer repeat (Baum et al., 1994). The central domain is also distinguished from the rest of the genome by the presence of the histone H3 variant CENP-A^{Cnp1} (Takahashi et al., 2000), which presumably confers a specialised structure or organisation to the central domain and may account for its atypical nuclease sensitivity. In contrast the outer repeat sequences are packaged in heterochromatin and display a regular nucleosomal ladder pattern on MNase digestion (Polizzi and Clarke, 1991, Takahashi et al., 1992).

Transcriptional silencing at centromeres

Genes placed in the vicinity of centromeric heterochromatin in mouse cells are found to exhibit variable states of expression (Butner and Lo, 1986, Festenstein et al., 1996). This

phenomenon, termed position effect variegation (PEV), was first described 1930 in *Drosophila* by Muller when a chromosomal inversion placing a wild type white gene (required for red eye pigmentation) adjacent to centromeric heterochromatin resulted in flies with a mixture of red and white patches of cells within the compound eye, due to expression or repression of the gene within individual cells. PEV has since been used by several groups to show that genes placed adjacent to centromeric heterochromatin are unstably repressed in *Drosophila* (Eissenberg et al., 1989, Henikoff et al., 1990, Karpen et al., 1994). Marker genes placed within fission yeast centromeres are also transcriptionally repressed (Allshire et al., 1994, 1995). When the *ade6*$^+$ gene is placed within the central core region of the fission yeast centromere, cells show reversible position effects on the *ade6*$^+$ gene, which can switch between alternate states of expression or repression (Allshire et al., 1994). As discussed above, fission yeast centromeres are composed of two distinct domains: the central domain (*cnt* + *imr*) upon which the kinetochore assembles and the outer repeat regions (*otr*), which are heterochromatic. The stability and magnitude of silencing imposed at the different sites across the two domains is not equivalent; in general, silencing is very strong in the *otr* repeats, but considerably less within the central domain (*cnt* and *imr*) (Allshire et al., 1995), which may reflect the different protein requirements or distinct functions of these two domains.

In fission yeast, cells with defects in silencing frequently show defective centromere function as assayed by chromosome mis-segregation (Allshire et al., 1995, Ekwall et al., 1995, 1996, 1999, Pidoux et al., 2003). Thus, transcriptional silencing of an inserted marker gene can be used as a read out for central domain function or heterochromatin integrity. The use of such transcriptional assays has led to the discovery of genes/proteins, which specifically alleviate silencing either at the central domain or at the outer repeat domain (Ekwall et al., 1999, Pidoux et al., 2003). Although exceptions exist, in general, proteins required for central core silencing are associated with the central domain and kinetochore function, whereas proteins encoded by genes that when mutated result in alleviation of outer repeat silencing are associated with the outer repeat domain and heterochromatin function (summarised in Figure 1-5). Here, heterochromatin is defined as chromatin containing histone H3 that is dimethylated on lysine 9 (H3K9me2) and is bound by Swi6, the heterochromatin protein 1 (HP1) equivalent in fission yeast.

Central domain and kinetochore proteins

Transcriptional silencing within the central domain is thought to reflect the formation of a functional centromere and kinetochore. Numerous screens have identified kinetochore proteins, such as Ams2, Mis6, Mis12, Sim4 and Mal2, which are specifically associated with the central core region as shown by chromatin immunoprecipitation (Saitoh et al., 1997, Goshima et al., 1999, Jin et al., 2002, Pidoux et al., 2003, Chen et al., 2003). Many of these

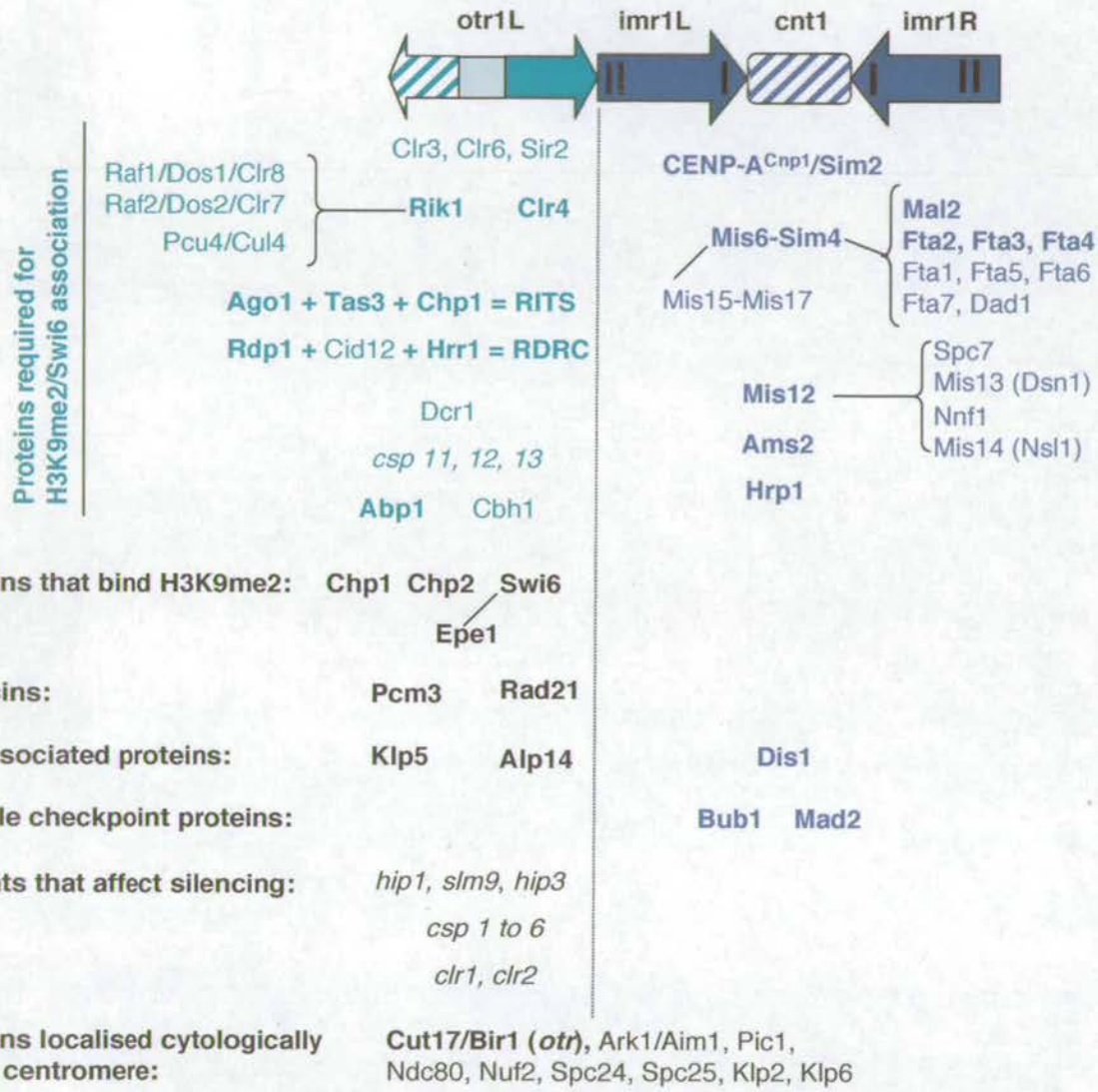


Figure 1-5. Proteins associated with the central domain or the outer repeat heterochromatin domain of the fission yeast centromere (adapted from Pidoux and Allshire, 2005)

Heterochromatin proteins are shown to the left and central domain proteins are shown to the right in purple. Proteins shown in green are required for H3K9me2 and Swi6 association with outer repeats. Proteins that are physically associated with either domain of the centromere by ChIP are shown in bold. Proteins that are known to be required for the proper function of either domain, but for which ChIP data has not been published are shown in regular font. Mutants that affect silencing at either domain are shown in italics. Only the left outer repeat of centromere 1 (*otrL*) is shown for simplicity and proteins associated with *otrL* are found in association with *otrR*. RITS is the RNAi-mediated Initiation of Transcriptional Silencing complex, RDRC is the RNA-Directed RNA polymerase Complex.

kinetochore mutants, including *sim4*, *mis6* and *mal2*, alleviate silencing at the central domain (Partridge et al., 2000, Jin et al., 2002, Pidoux et al., 2003). In addition, many of these mutants show reduced association of CENP-A^{Cnp1} with the central domain of the centromere (Takahashi et al., 2000, Pidoux et al., 2003, Chen et al., 2003). A common feature displayed by all of these kinetochore mutants is a disrupted MNase digestion pattern within the central domain, as assayed by the loss of the characteristic smear pattern that is replaced by a regular nucleosomal ladder pattern (Jin et al., 2002, Pidoux et al., 2003, Saitoh et al., 1997, Goshima et al., 1999, Chen et al., 2003, Takahashi et al., 2000). Although many proteins have been localised to the kinetochore and may be required for the association of CENP-A^{Cnp1} with centromeres, the precise role of each individual protein at the kinetochore has not yet been fully characterised (see Figure 1-3 for a comparison of the molecular organisation of the vertebrate, fission yeast and budding yeast centromere/kinetochore proteins).

CENP-A^{Cnp1} is the fission yeast equivalent of human CENP-A and is found associated with *cnt* and *imr* sequences at the centromere (Takahashi et al., 2000). The *cnp1-1* temperature sensitive mutation in CENP-A leads to unequal segregation of DNA during mitosis, where sister chromatids are capable of interacting with the spindle, are separated and moved to poles, but the fidelity of even segregation is greatly reduced. In *cnp1-1* mutants, the characteristic smear pattern of the *cnt* and *imr* is abolished and the disruption of centromeric chromatin occurs before chromosome mis-segregation is observed (Takahashi et al., 2000). This suggests that the presence of CENP-A^{Cnp1} contributes to the formation of a specialised chromatin in the central domain, which may be a prerequisite for equal segregation in the next mitosis.

The kinetochore proteins Mis6 and Mal2 were originally identified in mini-chromosome loss screens and *mis6* and *mal2* mutants were found to display defects in chromosome segregation (Takahashi et al., 1994, Fleig et al., 1996). Both proteins are specifically associated with the central domain by chromatin IP and *mis6* and *mal2* have been shown to alleviate central core silencing (Saitoh et al., 1997, Jin et al., 2002, Partridge et al., 2000). Sim4 (homologue of human CENP-H) is a kinetochore protein that was identified in a screen to isolate mutants that allowed the expression of an *arg3*$^{+}$ gene inserted at the central core (Pidoux et al., 2003). Sim4 is associated with the central core domain and is found in a complex with Mis6 (Pidoux et al., 2003, Hayashi et al., 2004). Alleles of *cnp1*$^{+}$ (called *sim2*$^{+}$) isolated from the screen were also found to alleviate central core silencing, indicating that the presence of CENP-A^{Cnp1} correlates with correct chromatin structure at the centromere (Pidoux et al., 2003). Both *sim4* and *mis6* mutants display disrupted centromere chromatin as assayed by MNase digestion and display reduced association of CENP-A^{Cnp1} with the central core (Pidoux et al., 2003, Takahashi et al., 2000). Moreover, Mis6 is required for the association of newly synthesised CENP-A^{Cnp1}-GFP at centromeres (Takahashi et al., 2000).

In addition, Mis6 is thought to act at G1-S, just after CENP-A^{Cnp1} mRNA levels peak (Saitoh et al., 1997, Takahashi et al., 2000). These observations support the model that Mis6, together with Sim4, may be acting as CENP-A^{Cnp1} specific loading factors.

The Sim4 complex that consists of Sim4, Mal2, Mis6, Mis15, Mis17, the DASH component Dad1 and a group of novel Fta1 to 7 proteins has also been identified (Liu et al., 2005). Fta2, Fta3 and Fta4 were found to be associated with the central core *cnt* and *imr* region of the centromere (Liu et al., 2005). In a separate study, Fta2 was shown play a role in bipolar chromosome attachment and is required for central core silencing (Kerres et al., 2006). Mis6 was previously shown to be in a complex with Mis15 and Mis17, both of which are found to associate with the centromere cytologically and by chromatin IP (Hayashi et al., 2004). *mis15* and *mis17* mutants show disrupted centromere chromatin by MNase digestion and have reduced CENP-A^{Cnp1} associated with the centromeres (Hayashi et al., 2004). Together with Mis6, Mis15 and Mis17 may act in a pathway to deliver CENP-A^{Cnp1} to the centromere.

Ams2 was originally identified as a multicopy suppressor of *cnp1-1* and is a GATA-like transcription factor, which is enriched at the centromere, along with other regions throughout the genome (Chen et al., 2003). *ams2Δ* cells fail to localise CENP-A^{Cnp1} in S phase and thus Ams2 is required for the replication coupled assembly of CENP-A^{Cnp1} as discussed below (Chen et al., 2003, Takahashi et al., 2005). Deletion of fission yeast Hrp1, which shows homology to the CHD (chromo-helicase/ATPase DNA binding) remodelling factor, gives rise to alleviation of silencing at *cnt* and Hrp1 also appears to associate with the central core in S phase by chromatin IP (Waldfridsson et al., 2005). Hrp1 deleted cells show unequal segregation of chromosomes in mitosis, which is consistent with a role in centromere function (Waldfridsson et al., 2005).

The kinetochore protein Mis12 was identified in the same mini-chromosome loss screen as Mis6 and is also required for the correct segregation of chromosomes (Goshima et al., 1999). Mis12 is a homologue of the kinetochore component Mtw1p in budding yeast, which is also required for accurate chromosome segregation (Goshima and Yanagida, 2000). Mis12 is bound to *cnt* and *imr* regions and the inner centromere-specific chromatin digestion pattern is abolished in *mis12* temperature sensitive mutants (Goshima et al., 1999). In budding yeast, Mtw1p functions in a complex with Nnf1p, Nsl1p and Dsn1p (reviewed by Chan et al., 2005). A similar complex was identified in *S. pombe* by genetic interactions and contains Mis12, Mis13 (Dsn1), Nnf1 and Mis14 (Nsl1), along with Spc7 (Obuse et al., 2004). Both Mis13 and Spc7 were confirmed cytologically to localise to the kinetochore and are bound to centromeric sequences *cnt* and *imr* (Obuse et al., 2004). Spc7 associates with the EB1 family member Mal3 that binds plus end microtubules and may facilitate interactions between the Ndc80, Mis12 and Sim4 complexes at the kinetochore-microtubule interface (Kerres et al., 2004, 2006). Mis14, which shows similarity to budding yeast kinetochore

protein Nsl1, also localises to the centromere and is required for the specialised nuclease digestion pattern at the centromere (Hayashi et al., 2004). However, both Mis14 and Mis12 are indispensable for the localisation of CENP-A^{Cnp1} to centromeres (Hayashi et al., 2004). Surprisingly, *mis12* mutants do not display alleviation of silencing at the central core (Dunleavy, E., unpublished observation). This suggests that the presence of CENP-A^{Cnp1} correlates with silent central domain chromatin, but that alleviation of silencing does not always correlate with the loss of the MNase smear pattern.

In human cells, hMis12 is also required for equal segregation of chromosomes and in cells depleted of hMis12, CENP-A localises normally to kinetochores, suggesting that like in fission yeast, hMis12 localises to kinetochores in a manner that is independent of CENP-A (Goshima et al., 2003). The human Mis12 complex was recently isolated and is composed of a discrete complex of kinetochore proteins hNsl1, hNnf1 and hDsn1 among other proteins (Cheeseman et al., 2004, Obuse et al., 2004, Kline et al., 2006). Like hMis12, hNsl1, hNnf1 and hDsn1 are required for accurate alignment and segregation of chromosomes in mitosis (Kline et al., 2006). In addition cells depleted of hDsn1 showed a 50% reduction in the association of CENP-A with the centromere, indicating that it may play a role in the localisation of CENP-A to the centromere (Kline et al., 2006). Human Mis12 also forms a stable complex with the centromeric heterochromatin proteins HP1α and HP1γ and double HP1 RNAi appears to affect the kinetochore localisation of hMis12 in HeLa cells (Obuse et al., 2004). These results require further confirmation but do suggest that the hMis12 complex may be acting as a bridge between heterochromatin and the domain where the kinetochore assembles.

Outer repeat domain and heterochromatin proteins

Observations from several studies initially showed that fission yeast centromeres, telomeres and the silent mating type loci are subject to transcriptional repression (Allshire et al., 1994, 1995, Thon et al., 1994, Nimmo et al., 1994). Mutations in genes involved in mating type switching such as *clr1*, *clr2*, *clr3*, *clr4*, *rik1* and *swi6* genes were first shown to alleviate silencing at the mating type loci (Egel et al., 1984, Thon and Klar, 1992, Ekwall and Ruusala, 1994, Lorentz et al., 1994, Thon et al., 1994). This prompted an investigation into whether genes that affect silencing at the mating type region, affected silencing at other silent chromosomal loci. Subsequently, the *clr4*, *rik1* and *swi6* genes, and to a lesser extent the *clr1*, *clr2* and *clr3* genes were shown to alleviate silencing at the outer repeats of *cen1* and at telomeres (Ekwall and Ruusala, 1994, Allshire et al., 1995). Mutants such as *clr4* and *rik1* that are defective in outer repeat silencing, display a high degree of lagging chromosomes at late anaphase, are sensitive to the microtubule (MT) destabilising drugs and display an elevated rate of loss of a non essential mini-chromosome (Allshire et al., 1995, Ekwall et al.,

1995, 1996). These phenotypes suggest that proteins that contribute to outer repeat silencing, play an important role in centromere function and chromosome segregation.

Chromodomain proteins: Swi6 and Clr4

Swi6 is a chromodomain protein and is the fission yeast counterpart of mammalian and *Drosophila* heterochromatin protein 1 (HP1) (Ekwall et al., 1995). Mutations in *swi6* lead to the alleviation of silencing at the centromeric outer repeats, the mating type locus and at telomeres (Ekwall and Ruusala, 1994, Thon et al., 1994, Allshire et al., 1995). Swi6 has been shown to localise to these three heterochromatic regions by immunostaining and FISH (Ekwall et al., 1995). Cells lacking Swi6 protein display an elevated loss of a mini-chromosome and have a high frequency of lagging chromosomes on late anaphase spindles (Ekwall et al., 1995). The localisation of Swi6 is dependent on Clr4 and Rik1 (Ekwall et al., 1996). Clr4 is the fission yeast homologue of *Drosophila Su(var)3-9*/ human SUVAR39H1 and is a histone methyltransferase that methylates histone H3 on lysine 9, providing a binding site for Swi6 (Rea et al., 2000, Bannister et al., 2000, Nakayama et al., 2001). Thus, *clr4Δ* cells lack histone H3 that is methlyated on lysine 9 (H3K9me2) at the outer repeats and are defective in Swi6 localisation, as they lack the Swi6 binding site (Nakayama et al., 2001). *swi6* mutants retain normal levels of H3K9me2, indicating that Swi6 acts downstream of H3K9me2 methylation (Nakayama et al., 2001).

Histone deacetylases

Methylation of H3K9 by Clr4 is preceded by a histone deacetylation step, involving the histone deacetylases (HDACs) Clr3, Clr6 and the NAD-dependent deacetylase Sir2 (Grewal et al., 1998, Shankaranarayana et al., 2003, Bjerling et al., 2002). Clr3 deacetylates lysine 14 of histone H3, whereas Sir2 acts to deacetylate lysine 9 of histone H3 (Shankaranarayana et al., 2003). Clr6 exhibits a broader specificity and deacetylates several of the lysines in the tails of histone H3 and H4 (Bjerling et al., 2002). All three HDACs are required for correct silent chromatin formation at outer repeat heterochromatin, the mating type locus and telomeres (Grewal et al., 1998, Shankaranarayana et al., 2003). Deacetylation of histone tails at the centromere plays an important role in centromere function as treatment of cells with the histone deacetylase inhibitor trichostatin A (TSA) leads to alleviation of silencing of a marker gene inserted at the outer repeats and gives rise to defects in chromosome segregation (Ekwall et al., 1997).

Rik1

As mentioned above, Rik1, along with Clr4, is required for Swi6 localisation to heterochromatin (Ekwall et al., 1996). Rik1 is a WD40 repeat containing protein related to both the DNA damage binding protein DDB1, and the large subunit of the mRNA cleavage and polyadenylation factor CPSF-A (Neuwald and Poleksic, 2000). Like *swi6Δ* and *clr4Δ*, *rik1Δ* cells alleviate silencing at the centromeric outer repeats, the mating type locus and at

telomeres (Ekwall and Ruusala, 1994, Thon et al., 1994, Allshire et al., 1995). Rik1 is found associated with the outer repeats and cells lacking Rik1 do not contain H3K9me2 at the centromeric outer repeats, mating type locus or telomeres (Nakayama et al., 2001, Sadaie et al., 2004). In addition, Rik1 associates with Clr4 and mutually affect each other's centromeric localisation, suggesting that Rik1 and Clr4 functionally co-operate to promote H3K9 methylation (Sadaie et al., 2004, Jia et al., 2005). Three independent studies identified Raf1 (also known as Dos1 or Clr8) and Raf2 (also known as Dos2 or Clr7) proteins, which were required for transcriptional silencing at the outer repeats, telomere and mating type loci and also for H3K9me2 at outer repeats and the localisation of Swi6 (Horn et al., 2005, Li et al., 2005, Thon et al., 2005). Raf1/Dos1 was found to interact with both Rik1 and Raf2/Dos1 and forms a complex that may regulate the interactions of the Rik1-Clr4 complex with outer repeat heterochromatin (Horn et al., 2005, Li et al., 2005). In addition, Rik1 and Clr7 (also called Raf2 or Dos2) were found in a complex with a cullin-dependent E3 ubiquitin ligase Pcu4 (also called Cul4) that is also essential for heterochromatin formation, however its precise role at the centromere has not yet been defined (Horn et al., 2005, Thon et al., 2005, Jia et al., 2005). It is possible, for example, that Rik1 acts as an adapter protein that facilitates the targeting of other factors for ubiquitination via Pcu4.

Chp1 and Chp2

The chromodomain protein Chp1 is localised to centromeres, telomeres and mating type loci in a pattern indistinguishable from that of Swi6 (Sadaie et al., 2004). However, *chp1Δ* cells alleviate outer repeat silencing, but not mating type or telomeric silencing (Partridge et al., 2000, Thon and Verhein-Hansen, 2000). In the absence of Chp1, cells show lagging chromosomes on late anaphase spindles and a high rate of chromosome loss and have reduced Swi6 associated with outer repeats (Doe et al., 1998, Sadaie et al., 2004). Like Swi6, Chp1 binds to H3K9me2 at the outer repeats and has been found as part of the RITS (RNA-induced initiation of transcriptional silencing) complex, together with the outer repeat associating protein Tas3 and Ago1 (Partridge et al., 2000, 2002, Verdel et al., 2004). Chp2 is a chromodomain protein related to Chp1 and Swi6, and *chp2Δ* cells weakly alleviate centromere, mating type and telomere silencing and Chp2 is localised at all three loci (Halverson et al., 2000, Thon and Verhein-Hansen, 2000, Sadaie et al., 2004). As is the case for Swi6, the association of Chp1 and Chp2 with the centromeres is dependent on Clr4 (Sadaie et al., 2004). Chp1 is required for the correct association of Chp2 and Swi6 with the outer repeats and experiments where *clr4*$^{+}$ was reintroduced into *chp1Δ clr4Δ* double mutants showed that Chp1 is required for the establishment of H3K9me2 at outer repeats (Sadaie et al., 2004). It has been observed that in *chp1Δ* cells, a residual level of H3K9me2 is retained and both Swi6 and Chp2 were shown to be responsible for the maintenance of this residual level of methylation at the centromere (Sadaie et al., 2004).

CENP-B homologues

Abp1, Cbh1 and Cbh2 are the fission yeast homologues of the human CENP-B, a protein that binds to the 17 bp box of a conserved class of centromeric α satellite repeats in a sequence specific manner (Murakami et al., 1996, Lee et al., 1997, Irelan et al., 2001, Baum and Clarke, 2000). Cells lacking either *abp1* or *cbh1* are found to alleviate silencing at the outer repeats, have reduced levels of H3K9me2 and reduced Swi6 binding at outer repeats (Nakagawa et al., 2002). Furthermore, the CENP-B homologues may be functionally redundant at the centromere, as double disruptants show a synergistic reduction in the association of Swi6 with the outer repeats (Nakagawa et al., 2002). Abp1 was found to bind to the outer repeat regions and it has been proposed that CENP-B homologues may act as site-specific nucleation factors which facilitate the association of Swi6 with centromeric heterochromatin (Nakagawa et al., 2002).

Centromeric suppressor of position effects (*csp*) mutants

Twelve *csp* (centromeric suppressor of position effects) mutants have been isolated which alleviate silencing of *otr* and *imr* (but not *cnt* nor at the telomeres nor at silent mating type loci) and display chromosome mis-segregation phenotypes (Ekwall et al., 1999). The mutants fall into two classes: temperature sensitive (*csp1* to *csp6*) and non-temperature sensitive (*csp7* to *csp13*); *csp8* and *csp10* are allelic to each other. *csp7* to *csp13* have been shown to lose methylation of lysine 9 and fail to recruit Swi6 and Rad21-cohesin at outer repeats; *csp9* is an allele of the RNAi component *ago1* (Volpe et al., 2002, 2003). *csp7* and *csp10* have been cloned in the laboratory (by Sharon White) and are alleles of the RNAi components *rdp1* and *cid12* , respectively. *csp1* to *csp6* show a weak alleviation of silencing and have more subtle effects on the levels of H3K9me2 and Swi6 at the centromere compared to non temperature sensitive *csp* mutants (M. Portoso, PhD Thesis, University of Edinburgh, 2005). It is likely that the other *csp* genes encode as yet undiscovered components of the RNAi pathway that contribute to heterochromatin assembly (described in detail below).

Other proteins required for heterochromatin integrity

Mutants defective in nucleosome assembly, such as *hip1*, *slm9* and *hip3*, the *S. pombe* homologues of the evolutionarily conserved HIRA-like (Hir) histone chaperone proteins, were also shown to have reduced transcriptional silencing at *otr* and are required for accurate chromosome segregation (Blackwell et al., 2004, Greenall et al., 2006). Epe1 is a jumonji C containing protein that was originally identified in a screen for factors that prevent the spreading of repressive chromatin beyond the natural borders of heterochromatin domains (Ayoub et al., 2003). Epe1 is localised to the outer centromeric repeats, mating type locus and telomeres, binds to Swi6 and its localisation at heterochromatic loci is dependent on Swi6 (Zofall and Grewal, 2006). It is proposed that Epe1 acts to facilitate the transcription of repeat elements at the centromere by negatively

affecting the stability of repressive heterochromatin complexes (Zofall et al., 2006). It has also been proposed that Epe1 may be a putative histone demethylase that could act by oxidative demethylation or as a hydroxylase that catalyzes a novel histone modification or the modification of a non-histone component (Trewick et al., 2005).

RNAi and heterochromatin assembly

Several lines of evidence in many systems now support a role for the RNA interference pathway in the formation of heterochromatin (reviewed by Lippman and Martienssen, 2004, Verdel and Moazed, 2005, see Figure 1-6). The RNAi pathway is generally thought to be responsible for post-transcriptional gene silencing (PTGS): in this process, a small (21-23 nucleotides) interfering RNA molecule (siRNA) triggers the degradation or translational inhibition of the homologous mRNA (Hannon, 2002). Factors involved in RNAi include an RNase III-like enzyme Dicer (*S. pombe* Dcr1), an RNA-dependent RNA polymerase (RdRP; *S. pombe* Rdp1) and an Argonaute protein (*S. pombe* Ago1) that has RNA 'slicing' activity (Irvine et al., 2006). In fission yeast, deletion of any of the genes encoding the RNAi components (Dcr1, Rdp1 and Ago1) results in alleviation of outer repeat silencing, accompanied by a decrease in H3K9me2 and Swi6 localisation (Volpe et al., 2002, Verdel et al., 2004, Motamedi et al., 2004). Fission yeast *RNAi* mutants (and also *clr4*Δ) have been shown to accumulate aberrant unprocessed transcripts from the outer repeat regions of the centromere that are normally rapidly turned over in wild type cells (Volpe et al., 2002, Sadaie et al., 2004). In addition, centromeric siRNAs derived from the centromeric repeats, which are detectable in the wild type, are not produced in these mutants (Motamedi et al., 2004, Hong et al., 2005, Li et al., 2005, Verdel and Moazed, 2005). RNAi components are also required for establishing heterochromatin assembly at the mating type locus, but are dispensable for its maintenance (Hall et al., 2002).

Components of the RNAi machinery are also required for the formation of centromeric heterochromatin in *Drosophila* (Pal-Bhadra et al., 2004). In experiments in chicken DT40 cells carrying human chromosome 21, Dicer deletion results in the accumulation of transcripts homologous to the human chromosome 21 (Fukagawa et al., 2004), which is similar to what is observed in fission yeast (Volpe et al., 2002). Fukagawa et al. (2004) also demonstrated that the vertebrate cells depleted of Dicer display defects in heterochromatin assembly, sister chromatin cohesion and chromosome segregation. Dicer deficient embryonic stem cells were also shown to accumulate centromeric transcripts (Murchison et al., 2005, Kanellopoulou et al., 2005), suggesting that the role of the RNAi machinery might be conserved in higher eukaryotes.

In fission yeast, siRNAs are produced by the action of Dcr1 on transcripts arising from the repetitive DNA at the outer centromeric repeats (see Verdel and Moazed, 2005). These

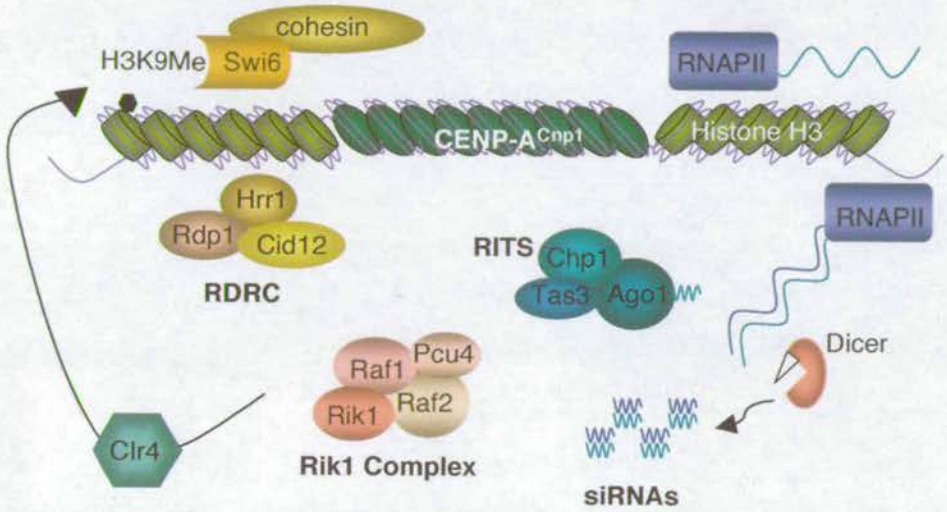


Figure 1-6. Model for RNAi and heterochromatin assembly in fission yeast (adapted from Sharon White)

Centromeric repeats are transcribed by RNA pol II and double stranded RNAs are produced and cleaved into siRNAs of 21-22 nucleotides in length by Dicer. Centromeric siRNAs are loaded into RITS (RNAi-mediated Initiation of Transcriptional Silencing) effector complex that targets the siRNAs back to the repetitive sequences and may trigger post transcriptional gene silencing either through mRNA degradation or translational repression. RITS may also act in concert with the histone methyltransferase Clr4 to bring about methylation of H3K9me2 and the binding of Swi6 and Cohesion, leading to transcriptional gene silencing. RITS is bound to the chromatin via the chromodomain of Chp1, which binds H3K9me2 and is dependent on Clr4. Clr4 is in turn recruited by the Rik1 complex. Rdp1 association with the outer repeats is dependent on RITS and acts to generate more dsRNAs from single stranded transcripts, that are processed by Dicer into siRNAs and serves to amplify signals to further recruit heterochromatin assembly factors. RDRC is the RNA-Directed RNA polymerase complex consisting of Hrr1, Rdp1 and Cid12.

centromeric siRNAs are loaded into an effector complex RITS (RNA-mediated Initiation of Transcriptional Silencing), which is comprised of Ago1, Tas3 and the chromodomain protein Chp1 (Verdel et al., 2004). All components of the RITS effector complex are necessary for heterochromatin formation, and like Rdp1, RITS components bind to the centromere, *mat* locus and telomeres (Noma et al., 2004, Verdel et al., 2004, Sugiyama et al., 2005). Rdp1 is also associated with two conserved proteins also required for outer repeat silencing, the RNA helicase Hrr1 and the putative polyA polymerase Cid12 in a complex termed RDRC (RNA-Directed RNA polymerase Complex) (Motamedi et al., 2004, Sugiyama et al., 2005). Deletion of any component of the RITS complex or Dcr1 or Clr4 abolishes Rdp1 localisation at the centromere (Sugiyama et al., 2005). It has also been shown that Rdp1 polymerase activity is essential for the generation of siRNAs, which, in turn mediates the recruitment of Clr4 and RITS to bring about dimethylation of histone H3 on lysine 9 (H3K9me2) at the centromere (Sugiyama et al., 2005). These observations have prompted the proposal that a self-enforcing RNAi loop mechanism couples siRNA generation with heterochromatin formation at the centromere (Noma et al., 2004, Sugiyama et al., 2005). Although *RNAiΔ* cells show reduced levels of H3K9me2, it is important to note that a residual level of H3K9me2 is retained in these mutants and it is proposed that Swi6 and Chp2, or a yet unknown factor, may contribute to the maintenance of this residual level of methylation (Sadaie et al., 2004).

It has recently been demonstrated that the tethering of the RITS subunit Tas3 to the RNA transcript of a normally active *ura4*$^+$ gene, can silence *ura4*$^+$ gene expression (Buhler et al., 2006). As the silencing of the *ura4*$^+$ gene is dependent on a functional RNAi pathway and the heterochromatin proteins Sir2, Clr4 and Swi6 this provides evidence for transcriptional gene silencing in fission yeast. Based on the observation that similar levels of RNA pol II are associated with active and silenced genes at the centromere, these experiments suggested that RNA pol II and its nascent transcripts are an integral component of heterochromatin and contribute to a co-transcriptional gene silencing (CTGS) mechanism (Buhler et al., 2006).

Role of heterochromatin at centromeres

Outer repeat heterochromatin is also required to attract the Cohesin complex which is distributed along the entire length of chromosomes, is enriched at centromeres and mediates sister chromatid cohesion in fission yeast (Bernard et al., 2001). In *S. pombe*, the Cohesin complex consists of four subunits Psm1, Psm3, Psc3 and Rad21 (called Smc1p, Smc3p, Scc3p and Scc1p respectively in *S.cerevisiae*) (Tanaka et al., 2000, Tomonaga et al., 2000). Homologues of these four proteins are found in yeast to humans and appear to be conserved in function (reviewed by Uhlmann, 2001). Anaphase is triggered by the cleavage of the Rad21 (Scc1p) subunit of cohesin, allowing sister chromatid separation (Nasmyth,

2002). The binding of Swi6 at the centromere is also required for the recruitment of a high density of the cohesin subunit Rad21 and is important for the maintenance of sister chromatid cohesion (Bernard et al., 2001, Nonaka et al., 2002). Cells lacking *swi6* are defective in centromeric cohesion between sister chromatids, but arm cohesion is maintained (Bernard et al., 2001, Nonaka et al., 2002). *rad21*^{ts} cells show a high incidence of lagging chromosomes even at the permissive temperature and *rad21 swi6* double mutants are synthetically lethal, as they lose both centromeric and arm cohesion (Bernard et al., 2001). Swi6 has also been shown to interact directly with the cohesin subunit Psc3 (Nonaka et al., 2002).

Cohesin at the centromere is thought to act as a type of 'glue' that perhaps holds sister kinetochores in a rigid back-to-back conformation and facilitates the proper interaction of the two kinetochores with microtubules emanating from opposite poles (reviewed by Bernard and Allshire, 2002). Thus, any defect in the formation of Swi6-heterochromatin results in the defective recruitment of Rad21, leading to defects in centromeric cohesion between sister chromatids, culminating in defects in chromosome segregation. Mutants defective in outer repeat silencing often show sensitivity to microtubule destabilising drugs and display lagging chromosomes in anaphase as they fail to make proper microtubule attachments due to the disrupted chromatin structure (Allshire et al., 1995, Ekwall et al., 1995, 1996). In mammalian cells, it has been shown that lagging chromosomes often result from the merotelic attachment of a single sister chromatid to kinetochore microtubules rooted in opposite poles, rather than to only one pole as normally occurs by metaphase (Cimini et al., 2001). Merotelic kinetochore orientation is a major limitation for accurate chromosome segregation and a potentially important cause of aneuploidy in human cells (Cimini et al., 2001, 2002). The presence of the heterochromatic outer repeats and maintenance of proper sister chromatid cohesion is likely to play a key structural role in co-ordinating the presentation of the kinetochore in the correct configuration that enables proper microtubule attachment at cell division.

Microtubule interactions and transient interactions at the kinetochore

The microtubule (MT)-associated proteins (MAPs) Dis1 and Alp14 have been shown to be associated with the *cnt* and *imr/otr* regions of the centromere respectively (Nakaseko et al., 2001, Garcia et al., 2001). Both proteins associate with the centromere in a mitosis dependent manner and the association of Alp14 is completely dependent on the presence of MTs (Nakaseko et al., 2001, Garcia et al., 2001). As Dis1 and Alp14 associate with inner and outer centromeric DNA respectively and also with plus end microtubules, these proteins may act as bridging factors allowing correct kinetochore-spindle attachments (Garcia et al., 2002). Klp2 is a kinesin kar3 family member that is homologous to the vertebrate MT-binding protein XMAP215/cnTOG and localises to the centromere in mitosis (Troxell et al.,

2002). Klp5 and Klp6 belong to the kinesin-8 family (homologous to the vertebrate kinesin MCAK) that also localise to the centromere during mitosis and Klp5 has been shown to be associated with the outer repeats (Garcia et al., 2002, West et al., 2001). The fission yeast DASH complex is transiently associated with kinetochores during mitosis and may act to secure the binding of kinetochores to the dynamic ends of microtubules, although it is not essential for viability (Liu et al., 2005).

The spindle assembly checkpoint (SAC) coordinates cell cycle progression and chromosome segregation by inhibition of the anaphase-promoting complex (cyclosome) until all kinetochores make proper attachments with the spindle. During early mitosis the spindle checkpoint components, such as the Mad and Bub proteins, accumulate at kinetochores that are not attached to the spindle (reviewed by Musacchio and Hardwick, 2002, Cleveland et al., 2003). In fission yeast, it is suggested that Bub1 recognises tension-less kinetochores, whereas Mad2 detects unattached kinetochores (Garcia et al., 2002). In accordance with this, Bub1 was found to associate with the central core only when the spindle checkpoint is activated (Toyoda et al., 2002). During early mitosis Mad2 localises at kinetochores that are not yet attached to the spindle and is found to bind to *cnt* and *imr* sequences even in a normal mitosis (Vanoosthuyse et al., 2004). Mis6 is also found in a complex with Mad2 and may facilitate the accumulation of Mad2, but not Bub1, at the kinetochore (Saitoh et al., 2005). Fission yeast homologues of the budding yeast outer kinetochore Ndc80 complex (Nuf2-Hec1-Spc24-Spc25) have been localised cytologically to the centromere (Wigg and Kilmartin, 2001, Nabetani et al., 2001). The Ndc80 complex is also required for the localisation of Mad2 to the kinetochore (Saitoh et al., 2005). The role of the Mis6-Sim4 and Ndc80 complexes in Mad2 accumulation are also evolutionarily conserved as human Mis6 (CENP-I) and the equivalent of the Ndc80 complex in humans (hNuf2-hHec1) have been shown to be required for the kinetochore accumulation of hMad2 and hMad1 (Martin-Lluesma et al., 2002, DeLuca et al., 2003, Liu et al., 2003).

Ark1 (also known as Aim1) is the Aurora B kinase homologue in fission yeast, which is required for spindle formation, chromosome segregation and cytokinesis (Petersen et al., 2001, Rajagopalan and Balasubramanian, 2002). Ark1 in fission yeast behaves as a chromosomal passenger protein and localises to the centromere in early mitosis and redistributes to the spindle midzone at anaphase, as occurs with the metazoan Aurora B-INCENP-Survivin complex (reviewed by Adams et al., 2001). In budding yeast, Ipl1p/Aurora senses the absence of tension and activates the spindle checkpoint (Biggins and Murray, 2002) whereas fission yeast Ark1/Aurora is required to activate the spindle checkpoint in response to unattached kinetochores (Peterson et al., 2001). Pic1 and Cut17/Bir1 are the fission yeast homologues of INCENP (inner centromere protein) and Survivin, respectively (Morishita et al., 2001, Leverson et al., 2002, Rajagopalan and Balasubramanian, 2002). Cut17 also shows a localisation pattern similar to the passenger

protein complex in higher eukaryotes and is found to associate with the outer repeats by chromatin IP (Morishita et al., 2001) (see Figure 1-3).

HISTONE VARIANTS AND CHROMATIN ASSEMBLY

The majority of chromatin assembly takes place immediately after DNA replication by a process that involves the initial deposition of histones H3 and H4 followed by the incorporation of two histone H2A-H2B dimers as a heteromeric tetramer to complete the nucleosome (reviewed by Akey and Luger, 2003). At the replication fork, histones from the parental nucleosomes are randomly distributed between the two daughter DNA strands and the remainder histones required to complete the histone octamer are synthesised *de novo* during the S phase of the cell cycle (reviewed by Krude and Keller, 2001). In contrast to the canonical histones, histone variants are usually expressed throughout the cell cycle and can be assembled into nucleosomes independent of DNA replication (reviewed by Henikoff et al., 2002). Histone variants are assembled into chromatin outside of replication to regulate the processes of DNA repair, recombination and transcription, thus histone variants provide a means of specifying alternative chromatin states (reviewed by Henikoff et al., 2004).

Histones are positively-charged, basic amino acids with an intrinsic affinity for the highly negatively-charged phosphate groups of DNA backbone. To ensure that chromatin assembly occurs in a regular and ordered manner, additional negatively charged molecules that protect the histones from the charged DNA, called histone chaperones, are required (reviewed by Philpott et al., 2000). Histone chaperones may act alone by promoting the deposition of histones onto DNA or may act in concert with ATP-dependent chromatin remodellers, such as ACF/CHRAC (ATP-utilising chromatin assembly and remodelling factor/ chromatin accessibility complex) or CHD1 (chromo-ATPase-helicase-DNA binding protein 1) to facilitate the assembly of periodic arrays of nucleosomes (reviewed by Haushalter and Kadonaga, 2003). Other ATP dependent chromatin remodelling complexes such as human RSC (remodelling and spacing factor) and budding yeast SWR1 have been shown to assemble chromatin in the absence of a core histone chaperone (Loyola et al., 2004, Mizuguchi et al., 2004). In addition, histone chaperone complexes that are specialised for either replication coupled assembly of canonical histones, such as H3 (also referred to as H3.1), or replication independent assembly of histone variants such as CENP-A, H3.3 and H2A.Z have been identified and are described below (reviewed by Polo and Almouzni, 2006).

Canonical H3 assembly

H3 (also referred to as H3.1), like the other canonical histones, is deposited in an S phase dependent manner (Franklin and Zweidler, 1977). More recently, H3.1 has been found in a complex with the chromatin assembly factor (CAF1) that stimulates the deposition of the replicative histone H3.1 in a DNA-synthesis dependent manner *in vitro* (Tagami et al., 2004). CAF1 is composed of three subunits (p150, p60, p48) and coordinates nucleosome remodelling with DNA replication in S-phase (Smith and Stillman, 1989) or in DNA repair (Gaillard et al., 1996) to ensure a faithful transition of chromatin from one cell cycle to the next. The CAF1 component p48 was originally identified as a retinoblastoma binding protein (RbAp48) and is a member of a variety of chromatin-related complexes, including histone deacetylase and nucleosome remodelling complexes (reviewed by Ridgway and Almouzni, 2000). CAF1 is recruited to the replication fork via interactions with the PCNA component of the DNA replication machinery (Shibahara and Stillman, 1999). Replication-coupling-assembly factor (RCAF) is a complex of anti-silencing factor 1 (ASF1) protein and specifically acetylated histones H3 and H4 and has been shown to act synergistically with CAF1 to mediate nucleosome assembly during replication (Tyler et al., 1999, 2001) or repair (Mello et al., 2002). CAF1 also contributes to the inheritance of heterochromatin states through DNA replication by maintaining a replication specific pool of heterochromatin protein 1 (HP1) available for binding to chromatin after replication and also by targeting the H3K9 methyltransferase SETDB1 (SET domain, bifurcated 1) to the replication fork (Quivy et al., 2004, Sarraf et al., 2004).

Histone H3.3 assembly

The replacement variant H3.3 differs from H3.1 in only four amino acid positions, but unlike canonical histone H3.1, which is deposited strictly during S phase, H3.3 is expressed and can be deposited throughout the cell cycle (Hendzel et al., 1990, Ahmad and Henikoff, 2002). The HIRA complex has been shown to specifically recognise the histone H3 variant H3.3 and facilitate DNA-synthesis-independent nucleosome assembly (Ray-Gallet et al., 2002, Tagami et al., 2004). HIRA is the human homologue of *Saccharomyces cerevisiae* Hir1p and Hir2p that were originally identified as repressors of histone expression (Spector and Osley, 1993) and have since been implicated in nucleosome assembly and the organisation of repressive chromatin (Kaufman et al., 1998). H3.3 is the only version of H3 retained in budding yeast and fission yeast, presumably as it can deposit both during bulk chromatin assembly in S phase and after replication, whereas H3.1 is replication dependent (reviewed by Malik and Henikoff, 2003, Ahmad and Henikoff, 2002).

The replication independent deposition of H3.3 is proposed to act as a marker of active transcription, as demonstrated in *Drosophila* KC cells where H3.3 is found enriched at transcriptionally active ribosomal gene arrays (Ahmad and Henikoff, 2002). In human cells shortly after transcription is induced, H3.3 is found enriched at artificially constructed

transgene arrays and H3.3 is found to replace H3 that is methylated on lysine 9 (H3K9me) which is a marker associated with repressed heterochromatin (Janicki et al., 2004). It has also been demonstrated in mouse and in fission yeast that H3.3 preferentially localises to promoter regions (Chow et al., 2005, Choi et al., 2005). It is also clear that in fission yeast, H3.3 is deposited preferentially at transcriptionally active euchromatic regions and that deletion of heterochromatin specific proteins such as Clr4 and Swi6 increases the replication independent deposition at heterochromatic regions, such as the silent mating type or centromeres (Choi et al., 2005). Further studies in *Drosophila* have demonstrated that transcriptional elongation is required to stimulate the deposition of H3.3 and it is suggested that the RNA polymerase II or factors associated with the RNA polymerase may assist in H3.3 assembly independent of replication (Schwartz and Ahmad, 2005, McKittrick et al., 2004). However, it is not clear whether it is alterations in chromatin structure that occur during transcription or transcription itself that determines the deposition of H3.3 in a process that is independent of replication. In addition in metazoa, H3.3 is relatively enriched in modifications associated with transcriptional activity and is deficient in H3K9me2 compared to H3.1 (McKittrick et al., 2004). However, it has recently been shown that oligonucleosomes containing H3.3 may also contain H3.1 nucleosomes and in this context, H3.1 is enriched in post-translational modifications associated with H3.3 and active transcription (Loyola et al., 2006). This suggests that post-translational marks on histone variants, and possibly canonical histones, are not simply intrinsic to the individual histones but are also influenced by the surrounding chromatin environment.

H2A.Z assembly

H2A.Z is a conserved histone H2A variant (reviewed by Malik and Henikoff, 2003). In budding yeast, H2A.Z is incorporated near silenced regions of the genome and antagonises the effects of gene silencing by heterochromatin (Meneghini et al., 2003). H2A.Z is thought to act as a marker for the active chromatin state by maintaining chromatin in a transcriptionally permissive 'open' state (Fan et al., 2002). In budding yeast, the SWR1 complex including Swr1p (Swi2/Snf2-related ATPase 1), a member of the SWI/SNF family of ATP-dependent nucleosome remodelling enzymes, can specifically assemble H2A.Z-H2B dimers on nucleosome arrays and remove pre-existing H2A-H2B dimers *in vitro* in an ATP dependent manner (Mizuguchi et al., 2003, Krogan et al., 2003, Kobor et al., 2004). Replacement of H2A with H2A.Z influences gene expression *in vivo*, indicating that the presence of a histone variant is sufficient to alter the functional state of the underlying chromatin (Redon et al., 2002, Adam et al., 2001). A similar mechanism occurs in transcription by the action of the FACT (Facilitates Chromatin Transcription) complex, which displaces an H2A-H2B dimer, which is thought to stimulate transcript elongation, and subsequently FACT replaces an H2A-H2B dimer after the passage of the RNA polymerase (reviewed by Formosa et al., 2003, Berlotserkovskaya et al., 2003). In budding

yeast, H2A.Z-H2B dimers have also been shown to associate with nucleosome assembly protein 1 (NAP1) (Mizuguchi et al., 2003). Nucleosome assembly protein 1 (NAP1) is a highly conserved H2A-H2B binding protein (Ito et al., 1996, Ishimi et al., 1987) that can reversibly remove and replace H2A-H2B dimers into nucleosomes during transcription *in vitro* (Levchenko and Jackson, 2004, Park et al., 2005).

Macro H2A is also a H2A variant, which is enriched on the human inactive X chromosome and may be a marker of transcriptional repression (Constanzi and Pehrson, 1998). H2ABbd (Barr body deficient) is associated with transcriptionally active domains and is mutually exclusive to macroH2A (Chadwick and Willard, 2001). H2A.X is a histone variant that becomes phosphorylated in nucleosomes flanking DNA double strand breaks and acts as a marker of DNA lesions (reviewed Redon et al., 2002). The mechanisms of macroH2A and H2ABbd assembly into chromatin remain to be determined, however the activity of a H2A phosphatase complex has been shown to reverse H2A.X phosphorylation and is associated with efficient recovery from DNA damage (Keogh et al., 2005). In both fission and budding yeast, all H2A is of the H2A.X variety, as defined by a C terminal motif (Malik and Henikoff, 2003).

Histone H3 variant CENP-A

One hallmark of functional centromeres is the presence of CENP-A, the histone H3 variant that is specifically found in centromeric nucleosomes (Palmer et al., 1991, Sullivan et al., 1994). CENP-A homologues are found in all eukaryotes and are tightly associated with kinetochore activity and it is thought that this highly conserved histone H3 variant may be the primary determinant of centromere identity. When CENP-A is deleted or mutated, normal chromosome segregation is disrupted and the localisation of most other kinetochore components is abolished (Stoler et al., 1995, Buchwitz et al., 1999, Howman et al., 2000, Takahashi et al., 2000, Blower and Karpen, 2001).

Human CENP-A shares approximately 57% identity with histone H3 and this homology is restricted to the C terminal portion, as the CENP-A N terminus is unique and highly divergent among species (Sullivan et al., 1994, Figure 1-7). *In vitro* CENP-A has been shown to replace histone H3 in centromeric nucleosomes, where CENP-A nucleosomes can be formed from purified CENP-A and histones H2A, H2B and H4 and can replace both copies of histone H3 (Shelby et al., 1997, Yoda et al., 2000). In addition when viewed using atomic force microscopy, these homotypic CENP-A nucleosomes form conventional 'beads on a string' and are virtually indistinguishable from H3-containing nucleosomes (Yoda et al., 2000). The greatest divergence in CENP-A and histone H3 is in the N terminal sequence and, in fact, it is the N terminus that is most divergent among CENP-A homologues in

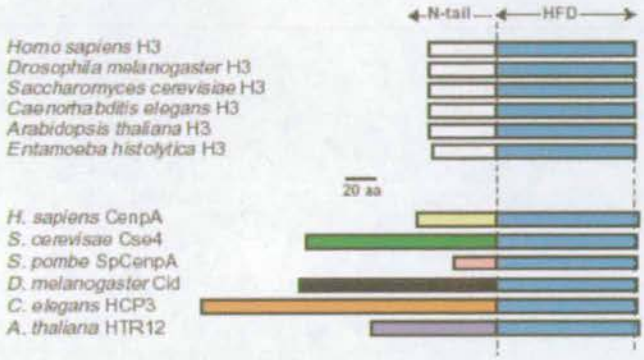


Figure 1-7. Schematic of canonical histone H3 compared with histone H3 variant CENP-A (from Malik and Henokoff, 2003).

The N-terminal tails of H3 are almost invariant, whereas the N-terminal tails of CENP-A are highly divergent and vary greatly in size (from 20 to 200 amino acids) and sequence across species. The C terminal histone fold domain (HFD) of human CENP-A shows approximately 57% identity with the histone fold domain of H3.

different species. Despite this however, it is the globular C terminus of CENP-A that determines targeting to the centromere (Sullivan et al., 1994). Although, the precise mechanism of CENP-A targeting to and assembly only at the centromere remains to be determined, candidate mechanisms are discussed below. It is possible that CENP-A targeting occurs and CENP-A chromatin is propagated via a combination of the mechanisms discussed below.

MECHANISMS OF CENP-A CHROMATIN ASSEMBLY

The presence of CENP-A itself

The best candidate as the epigenetic mark, which is recognised by factors that assemble the centromeric chromatin, is CENP-A itself. This hypothesis would imply that the presence of CENP-A containing nucleosomes alone is sufficient to assemble a functional kinetochore. As with the rest of the genome, parental CENP-A nucleosomes are partitioned equally into daughter chromatids during DNA replication (Shelby et al., 2000, Sullivan, 2001). The presence of parental CENP-A nucleosomes inherited by DNA replication itself may be the epigenetic factor that acts as the mark to direct chromatin assembly or to recruit a remodelling factor to the kinetochore after S phase. Alternatively, histone H3 nucleosomes could be deposited temporarily as a 'placeholder' for CENP-A nucleosomes, from which the histone H3 is actively removed and replaced with CENP-A after replication has occurred (Sullivan, 2001). To date there is no evidence to support the model that histone H3 is removed and replaced by CENP-A.

Studies of CENP-A over-expression in many systems have shown that when CENP-A is in excess in the cell it can localise ectopically to euchromatin (Collins et al., 2004, Henikoff et al., 2000, Van Hooser et al., 2001, Heun et al., 2006). However, investigations into whether this ectopic CENP-A alone is sufficient to form a functional centromere have given conflicting results. In human tissue culture cells, over-expressed CENP-A was incorporated into non-centromeric regions, could recruit the inner kinetochore protein CENP-C but could not produce functional ectopic kinetochores (Van Hooser et al., 2001). In a more recent study, in both developing flies and fly tissue culture cell lines, over-expression of CENP-A^{CID} resulted in what appeared to be kinetochore formation at ectopic loci that may be capable of making microtubule attachments (Heun et al., 2006). However, not all sites of ectopic CENP-A^{CID} incorporation were capable of the recruitment of kinetochore components, indicating that the presence of CENP-A^{CID} is not absolutely sufficient for the formation of a fully functional centromere and suggests that kinetochore proteins may be limiting in the cell.

Structural aspects of CENP-A

Human CENP-A consists of a C terminal domain that shows high homology to the C

terminal portion of histone H3 and a highly divergent N terminal domain (Sullivan et al., 1994). By constructing chimeric molecules composed of CENP-A and H3 N and C terminal domains, it was demonstrated that the C terminal histone fold domain of CENP-A is itself sufficient for targeting to the centromere (Sullivan et al., 1994). Similarly, a centromere-targeting element within the histone fold domain of CENP-A^{CID} has been described in *Drosophila* (Vermaak et al., 2002). In budding yeast, mutations in the histone fold domain of *CSE4* abolished centromere function (Keith et al., 1999) and in *Arabidopsis thaliana* the histone fold domain is sufficient to target CENP-A^{HTR12} to centromeres (Lermontova et al., 2006).

More recently, the region of CENP-A necessary for CENP-A targeting within the histone fold domain was assigned to a discrete motif called the CENP-A targeting domain (CATD) (Black et al., 2004). Replacement of the analogous region of H3 with the CATD motif was sufficient to restrict H3 targeting to the centromere in a replication independent manner. It was also demonstrated using deuterium exchange coupled with mass spectroscopy that the CENP-A-histone H4 tetramer is conformationally more rigid in structure than H3-H4 tetramers and this region included the CATD region (Black et al., 2004). Thus, nucleosomes containing CENP-A and H4 are biochemically and structurally more compact than H3-H4 nucleosomes, which together with the *cis*-acting CATD may act to mark the site for new CENP-A deposition.

Timing of centromere replication or CENP-A expression

Initially, centromeres were thought to be late replicating which lead to the idea that timing was important in propagating CENP-A chromatin at centromeres (Csink and Henikoff, 1998). In *Drosophila*, the timing of replication of centromeres was found to vary from early- to late- S phase (Ahmad and Henikoff, 2001, Sullivan and Karpen, 2001). In HeLa cells, CENP-A associated sequences replicate in mid- to late-S phase (Shelby et al., 2000). However, the timing of replication of centromeric sequences was found to overlap with that of other H3-containing regions of the genome in both fly and human cells (Sullivan and Karpen, 2001, Shelby et al., 2000, Blower et al., 2002). In addition, centromeres were shown to replicate asynchronously and there is no time in S phase when only centromeres are being replicated (Shelby et al., 2000, Ahmad and Henikoff, 2001) suggesting that specific CENP-A incorporation in late-S phase is unlikely to be controlled by timing of replication of centromeric DNA.

It has also been shown in human and flies that CENP-A assembly can take place in the presence of aphidicolin, a DNA replication inhibitor (Shelby et al., 2000, Ahmad and Henikoff, 2001). Thus, unlike histone H3.1, which is incorporated into chromatin in early S phase during replication, newly synthesised CENP-A can be loaded onto centromeres in G2 by a replication independent mechanism (Shelby et al., 2000). In addition, it was also

demonstrated that newly produced CENP-A^{CID}-GFP can also localise to centromeres in S phase with a similar efficiency to targeting in G2 in *Drosophila* Kc cells (Ahmad and Henikoff, 2001). Similarly, the incorporation of CENP-A in G2 has been demonstrated in *Arabidopsis thaliana* and both S phase dependent and G2 dependent CENP-A^{Cnp1} deposition pathways have been described in fission yeast (Takahashi et al., 2005, Lermontova et al., 2006). It is possible that CENP-A could be deposited at all stages of the cell cycle to ensure that it is constantly replenished at centromeres, but may have a preference for assembly in G2. This may provide a mechanism that distinguishes CENP-A assembly from that of the canonical histones that are specifically incorporated at replication. One proposed model for the replication independent loading of CENP-A suggests that during S phase, parental CENP-A nucleosomes are randomly distributed between the two daughter strands and H3 is temporarily placed opposite 'CENP-A sites', which are then subsequently replaced in G2 (Shelby et al., 2000, reviewed by Sullivan, 2001, reviewed by Henikoff and Dalal, 2005). A similar mechanism has been shown for the replacement of H3 with the variant H3.3 at active genes as previously described (Ahmad and Henikoff, 2002). Thus, replication-independent nucleosome assembly may be essential to maintain the specificity of CENP-A incorporation.

Uncoupling CENP-A mRNA expression from normal histone expression was also proposed to be an important mechanism for CENP-A targeting (Shelby et al., 1997), which would facilitate the preferential incorporation of CENP-A at a time when concentrations of potentially competitive histone H3 are diminished. In mammalian cells, CENP-A mRNA and protein was shown to begin to accumulate in S phase and peak in G2 (Shelby et al., 1997, Shelby et al., 2000). Indeed, restricting CENP-A expression to S phase abolished targeting to the centromere and incorporation of CENP-A becomes non centromere-specific (Shelby et al., 1997). However, as centromeric DNA is replicated during S phase with bulk chromatin (Sullivan and Karpen, 2001, Shelby et al., 2000, Blower et al., 2002), it is unlikely that timing of CENP-A expression is the mechanism of centromere specific CENP-A chromatin assembly as it is not made until later in the cell cycle. Uncoupling of CENP-A^{Cnp1} expression from that of H3 also occurs in fission yeast, CENP-A^{Cnp1} mRNA peaks in G1-S, before the peak of maximal histone H3 expression in S phase (Takahashi et al., 2000, 2005). Fission yeast centromeres are replicated in early S phase (Kim et al., 2001, 2003) as they are in budding yeast (McCarroll and Fangman, 1988). This suggests that increasing the concentration of CENP-A^{Cnp1} relative to H3 at the start of S phase and coordinating this increase with the timing of centromere replication may influence CENP-A^{Cnp1} assembly in yeast. Remarkably, when the CENP-A^{CID} promoter was used to drive the expression of yeast, worm and human CENP-A-GFP fusions, specific incorporation at pericentric heterochromatin surrounding fly and human centromeres was observed (Henikoff et al., 2000). In contrast, expression of H3-GFP under the control of the CENP-A^{CID} promoter, was found to give rise to euchromatic deposition of H3-GFP in flies (Henikoff et al., 2000).

These results suggest that CENP-A-like proteins from different organisms have an intrinsic affinity for pericentromeric heterochromatin, which is not controlled by the CENP-A^{CID} promoter and that may help sequester them to centromeres. Perhaps this affinity for pericentric heterochromatin is due to the rigidity of the CENP-A nucleosome as described by Black et al. (2004).

CENP-A specific assembly factors

The propagation of centromere identity may also be determined by the action of CENP-A assembly factors, which facilitate the specific incorporation of CENP-A into centromeric nucleosomes. Recently, the CENP-A nucleosome associated complex (NAC) has been identified in HeLa cells, consisting of three new human centromere proteins CENP-M, CENP-N, CENP-T, along with CENP-U(50), CENP-C and CENP-H (Foltz et al., 2006). Members of the complex were shown to localize to the centromere and are required for kinetochore integrity (Foltz et al., 2006), however the role of these proteins in CENP-A chromatin assembly has not been investigated. Seven new CENP-A nuceolsome distal (CAD) centromere components (CENP-K, CENP-L, CENP-O, CENP-P, CENP-Q, CENP-R and CENP-S) were found to assemble on the CENP-A NAC and are also involved in kinetochore integrity and may contribute to CENP-A chromatin assembly in chicken DT-40 cells (Foltz et al., 2006, Okada et al., 2006). In fission yeast, CENP-A^{Cnp1} can be incorporated in two distinct pathways that operate in S phase and G2 cells (Takahashi et al., 2005) and a number of proteins and complexes have been implicated in these deposition pathways. Many of the proteins required for CENP-A^{Cnp1} association in fission yeast appear to play a conserved role in higher eukaryotes.

In fission yeast *mis6* mutants, the localisation of CENP-A^{Cnp1} to the centromere is greatly diminished (Takahashi et al., 2000). Mis6 is found in a complex with Sim4 and *sim4* mutants also show a reduction in CENP-A^{Cnp1} association with the centromere (Pidoux et al., 2003). Moreover, Mis6 is required for the incorporation of newly synthesized CENP-A^{Cnp1}-GFP to the centromere (Takahashi et al., 2000). The budding yeast Mis6 homologue, Ctf3p, however is not required for the loading of CENP-A^{Cse4p} (Measday et al., 2002). Earlier studies on CENP-I, the vertebrate homologue of Mis6 and a Sim4 related protein CENP-H, revealed that neither were required for the stable association of CENP-A with the centromere (Goshima et al., 2003, Nishihashi et al., 2002). However, a more recent study has shown that depletion of a complex containing CENP-I and CENP-H in chicken DT-40 cells results in failure to incorporate newly synthesized CENP-A (Okada et al., 2006). Fission yeast Mis6 is found in a complex with Mis15 and Mis17 proteins and both Mis15 and Mis17 are required for the correct localisation of CENP-A^{Cnp1} (Hayashi et al., 2004).

The fission yeast Mis16-Mis18 complex is also required for CENP-A localisation to the centromere (Hayashi et al., 2004). The function of Mis16 appears to be conserved in

vertebrates as siRNA knockdown of both the human Mis16 homologues, RbAp46 and RbAp48 (human retinoblastoma binding proteins) disrupt the localisation of CENP-A (Hayashi et al., 2004). In addition, *in vitro* analysis suggests that *Drosophila* RbAp48 can assemble CENP-A^{CID} chromatin (Furuyama et al., 2006). However, these results require confirmation *in vivo* and did not demonstrate specificity of RbAp46 for CENP-A^{CID} over H3 or other histones. Also, RbAp46/RbAp48 have also been reported as members of chromatin modifying complexes that have histone deacetylase, histone acetylase and nucleosome positioning activities (Loyola and Almouzni, 2004). For example, RbAp48 is a component of the human chromatin assembly factor 1 (CAF-1) complex which functions in replication coupled assembly and is also found complexed to HIRA to facilitate transcription-coupled assembly (Tagami et al., 2004). Interestingly, fission yeast Mis16 and Mis18 appear to be required to maintain histone H3 and H4 at the central domain in a hypoacetylated state (Hayashi et al., 2004). Deletion of the fission yeast Hrp1 protein, which shows homology to the CHD (chromo-helicase/ATPase DNA binding) remodelling factor, also appears to result in a subtle increase in levels of acetylation of histone H3 and H4 and reduction in CENP-A^{Cnp1} association with the centromere (Waldfridsson et al., 2005). Thus, hypoacetylated histones may be a prerequisite for CENP-A^{Cnp1} assembly into chromatin at centromeres through the action of histone deacetylase complexes. It is not clear, however, whether this effect is due to an increase in the actual levels of H3 and H4 in the central domain, but these studies do demonstrate a change in the integrity of central domain chromatin has occurred.

Ams2 is a GATA-like transcription factor and has also been implicated in the association of CENP-A^{Cnp1} at centromeres (Chen et al., 2003). Deletion of *ams2*$^{+}$ results in diminished CENP-A^{Cnp1} at the centromere, however it is not lethal to the cell (Chen et al., 2003). In addition, Ams2 is required for the transcriptional activation of the histone genes during the S phase and the cell cycle dependent accumulation of histone mRNAs is abolished in *ams2Δ* cells (Takahashi et al., 2005). It is proposed that Ams2 plays a role in the S phase-coupled loading of CENP-A^{Cnp1}, possibly through the transcriptional upregulation of the expression of the histone genes in S phase when the histone proteins are required for deposition (Takahashi et al., 2005). As CENP-A^{Cnp1} can also be deposited at centromeres in G2, it is this alternative CENP-A assembly pathway that permits *ams2Δ* cells to be viable (Takahashi et al., 2005). As Ams2 associates with the centromere as assayed by chromatin IP (Chen et al., 2003), it is possible that Ams2 plays a direct role in remodelling centromeric chromatin to facilitate CENP-A^{Cnp1} assembly. However, as Ams2 has an effect on the levels of H3 and H4 relative to CENP-A^{Cnp1}, the affect on CENP-A^{Cnp1} association with centromeres could be indirect and may effect the interpretation of these results. As *mis6-302 hrp1Δ* double mutants show reduced growth compared to single mutants alone and *ams2Δ hrp1Δ* double mutants grow as well as single mutants (Waldfridsson et al., 2005), it is plausible that Hrp1 is acting in the Ams2-mediated replication-coupled CENP-A^{Cnp1} deposition pathway.

In budding yeast, the conserved Spt4p transcription factor restricts the localisation of CENP-A^{Cse4p} to the centromere and is required for chromosome segregation and kinetochore integrity (Crotti and Basrai, 2004, Basrai et al., 1996, Kerscher et al., 2003). In addition, complexes containing Spt4p are known to play a role in chromatin assembly and transcription elongation (reviewed by Winston et al., 2001). The budding yeast chromatin assembly factor Cac1p (equivalent to human CAF1) and the histone regulatory protein Hir1p (equivalent to human HIRA) are required for the association of Spt4p with the centromere (Crotti and Basrai, 2004). Moreover, *cacΔ hirΔ* double mutants display disrupted centromeric chromatin structure and also mis-localisation of CENP-A^{Cse4p} to euchromatin, but the localisation of CENP-A^{Cse4p} at the centromere is unaffected (Sharp et al., 2001). It is not known if transcription factors, such as Spt4p or Ams2, play a conserved role in CENP-A chromatin assembly but they do provide a link between centromeric chromatin assembly and transcription.

CENP-A association with centromeres via transcription?

There is now increasing evidence that transcription across the centromeric repeats may contribute to centromere formation and may be important for the deposition of CENP-A. Early work in newt lung epithelial cells demonstrated cytologically by electron microscopy, showed that the kinetochore contained an RNA component that was abolished on RNase treatment (Rieder, 1979). More recent analysis of human neocentromeres and studies of the CENP-A binding region in rice show that centromeres can contain active genes and are competent for transcription (Saffery et al., 2003, Nagaki et al., 2004, Yan et al., 2006). In maize, centromeric transcripts have been found to co-immunoprecipitate with CENP-A, suggesting that RNA may be an integral component of the centromere or may play a role in CENP-A assembly (Topp et al., 2005). In fission yeast, the GATA-like transcription factor Ams2 is required for CENP-A^{Cnp1} localisation to the centromere in S phase and has been shown to be associated with the centromeric sequences by chromatin IP (Chen at el., 2003). Recent data has shown that murine minor satellite repeats at the centromere accumulate transcripts ranging from 100 to 500 nucleotides long under conditions of stress and differentiation (Bouzinba-Segard et al., 2006). These transcripts co-localise with DAPI-dense foci and forced expression of a small 120 nucleotide non-coding RNA gives rise to misalignment of chromosomes in mitosis and defects in sister chromatid cohesion, suggesting that the transcripts have a role in centromere function. Interestingly, expression of Xist RNA is sufficient to promote the incorporation of the histone variant macro H2A1 specifically at the inactive X chromosome (Rasmussen et al., 2000). Moreover, the histone H3 variant H3.3 is deposited at active regions of the genome via transcription (Schwartz and Ahmad, 2005, McKittrick et al., 2004, Tagami et al., 2004). This suggests that an RNA transcript, either by acting alone or coupled to the transcription process, might facilitate the deposition of a histone variant to a specific region of the genome (Topp et al., 2004).

Interestingly, studies in human, flies and fission yeast have also shown that CENP-A nucleosomes at the centromere are interspersed with H3 nucleosomes that are methylated at K3 (H3K4me2) (Sullivan and Karpen, 2004, Cam et al., 2005, Lam et al., 2006), which is a mark of active transcription (Santos-Rosa et al., 2002). The H3K4me2 nucleosomes at the centromere may be as a result of transcription or may act to recruit the transcription machinery to the centromere. Alternatively, the H3K4me2 nucleosomes may act as 'placeholders' inserted during S phase to mark the site of CENP-A assembly. It is also proposed that H3K4me2 nucleosomes may contribute to the three dimensional structure of the centromere, making it structurally more rigid and help distinguish the centromere from the rest of the genome and push CENP-A nucleosomes to the outer face of the centromere to facilitate interactions with other kinetochore proteins and spindle microtubules (Sullivan and Karpen, 2004, Schueler and Sullivan, 2006, Figure 1-8).

The fact that CENP-A and specifically marked H3 are found at centromeres has lead to the suggestion that distinct combinations of histone modifications of CENP-A and H3, signify centromeric chromatin or 'centrochromatin' and may specify the site of kinetochore assembly (reviewed by Dunleavy et al., 2005). The only known CENP-A post-translational modification is the phosphorylation of serine 7 during mitosis in mammalian cells by the Aurora protein kinases (Zeitlin et al., 2001a, 2001b, Kunitoku et al., 2003). However, mutation of the serine 7 phosphorylation site does not affect targeting of CENP-A to the centromere but does result in defects in kinetochore function, chromosome alignment and cytokinesis (Zeitlin et al., 2001b, Kunitoku et al., 2003). Other post-translational modifications of CENP-A remain to be identified and may be key to the regulation of its assembly into chromatin.

Other possible mechanisms that contribute to CENP-A chromatin assembly

Other mechanisms that might facilitate the loading of CENP-A at centromeres include a biomechanical model where the tension created between active sister kinetochores and the pulling forces provided by microtubule attachment are the epigenetic signals required for new CENP-A incorporation (Mellone and Allshire, 2003). In this way, it is the presence of a functional kinetochore with proper microtubule attachments that marks the site of CENP-A incorporation and ensures CENP-A is replenished in the following cell cycle. Such a model would explain why so many kinetochore mutants affect CENP-A association with centromeres.

In budding yeast, proteolysis acts to restrict the localisation of CENP-A^{Cse4p} to the centromere and prevents the localisation of excess CENP-A^{Cse4p} to euchromatin (Collins et al., 2004). In addition this study showed that CENP-A^{Cse4p} already bound to the centromere is protected from this degradation pathway, suggesting that once incorporated into the

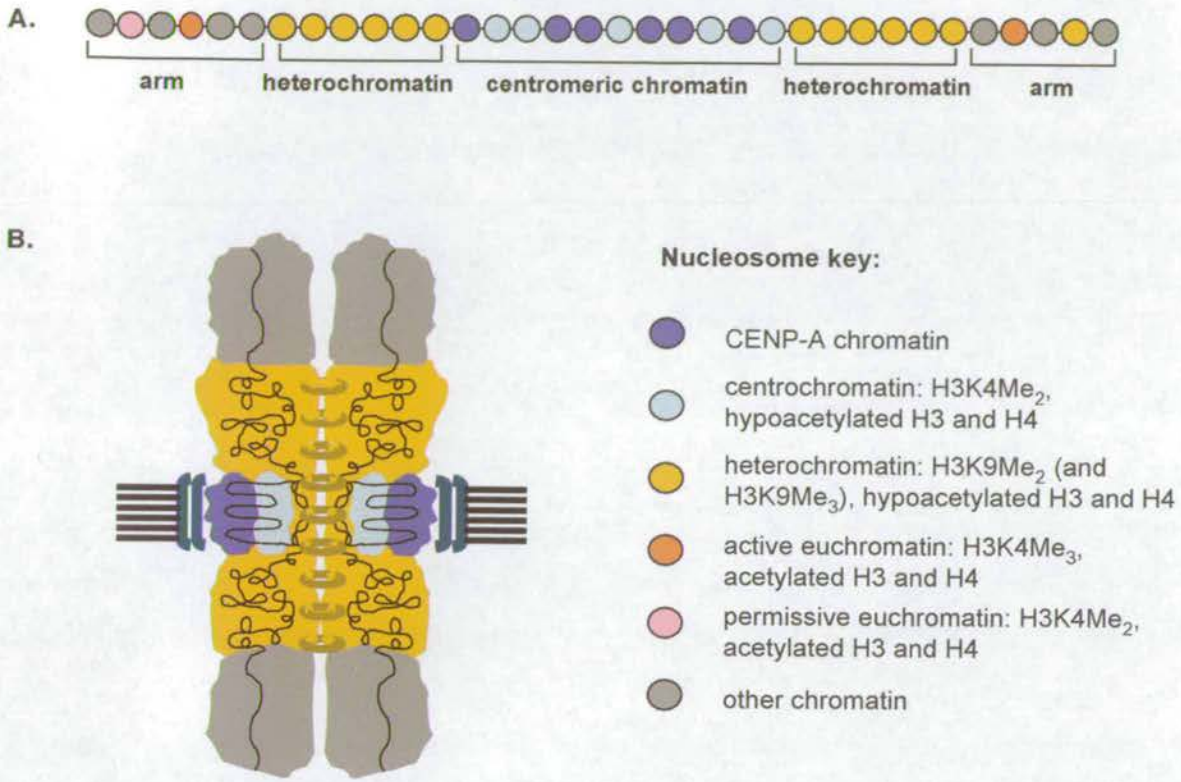


Figure 1-8. Proposed three dimensional organisation of the centromere in *D. melanogaster* and humans (from Dunleavy et al., 2005).

A. Diagrammatic representation of a linear chromatin fibre. Centromeric chromatin is marked by the presence of CENP-A nucleosomes (purple) interspersed with nucleosomes containing H3 that is dimethylated on lysine 4 (H3K4diMe, in pale blue). Blocks of heterochromatin (orange) flank the centromeric domain that are marked by hypoacetylation of histones H3 and H4 and H3K9Me (di and tri methylated) (shown in Sullivan and Karpen, 2004, Blower et al., 2002).

B. Model of chromatin organisation of condensed mitotic chromosome at metaphase. The 3D structure may result from coiling of the linear DNA fibre represented in **A**, resulting in stacks of CENP-A nucleosomes (purple) sitting on a platform of H3K4diMe nucleomsomes (pale blue), embedded in H3K9Me heterochromatin (orange). Stacks of CENP-A nucleosomes (purple) are oriented to the poleward face of the chromosomes where they can interact with other kinetochore proteins and spindle microtubules. Heterochromatin, marked by H3K9Me is found in a domain between sister centromeres and may function to recruit cohesion proteins (brown rings) that are maintained until sister chromatid separation at anaphase. The heterochromatin domain and the domain of H3K4diMe nucleosomes may function by giving structural rigidity to the centromere and push the CENP-A domain toward the outside of the chromosome (see also Sullivan and Karpen, 2004, Blower et al., 2002, Schueler and Sullivan, 2006).

specialised nucleosomes at centromeres, CENP-A^{Cse4p} is stable. A separate system, dependent on the Rad53p kinase that degrades excess soluble histones has also been described in budding yeast (Gunjan and Verreault, 2003). Whether proteolysis is a mechanism used to restrict the localisation of CENP-A to centromeres of other organisms remains to be investigated. It has also been demonstrated using sperm chromatin and a cell-free extract derived from *Xenopus* eggs, that endogenous DNA damage and base excision repair proteins such as UNG2 (uracil DNA glycosylase) may play a role in CENP-A assembly *in vivo* (Zeitlin et al., 2005).

SUMMARY

In summary, although many kinetochore components have been identified, little is known about specific factors that interact with CENP-A and how they influence or direct its assembly into chromatin at centromeres. In this thesis, I have performed a screen in fission yeast to isolate mutants that alleviate both central core and outer repeat silencing at centromeres. The *cos* mutants may provide a link between CENP-A^{Cnp1} chromatin assembly and the integrity of the flanking heterochromatin at centromeres. In addition, I have demonstrated that the conserved nuclear protein Sim3 is required for the deposition of CENP-A^{Cnp1} at fission yeast centromeres in interphase. Sim3 acts as a CENP-A^{Cnp1} chaperone and may contribute to CENP-A^{Cnp1} chromatin assembly at other stages of the cell cycle. Sim3 is homologous to the histone binding proteins mammalian NASP and *Xenopus laevis* N1/N2 and the role of *Drosophila melanogaster* DmNASP in CENP-A^{CID} chromatin assembly is also investigated.

CHAPTER 2

MATERIALS AND METHODS

2.1 GENERAL SOLUTIONS AND MEDIA

PBS per litre:
10 g NaCl
0.25 g KCl
1.43 g Na_2HPO_4
0.25 g KH_2PO_4
Autoclaved

TE:
1 mM EDTA
10 mM Tris-HCl, pH 8
Autoclaved

20X TBE per litre:
Tris Base 216.0 g
Boric Acid 110.0 g
80 ml 0.5 M EDTA, pH 8

Fission Yeast Media

All solutions were made up to the final volume with distilled H_2O. All the following solutions were autoclaved, unless otherwise stated.

PMG Agar (1L):
3.0 g Potassium Hydrogen Phthallate
2.2 g Di-sodium orthophosphate
3.75 g Glutamic acid
20 g D-Glucose anhydrous (Fisher Scientific)
Vitamins 1000X 1 ml
Minerals 10,000X 0.1 ml
50X Salts 20 ml
Agar 20 g (OXOID)

PMG liquid (1L):
as above without addition of Agar

YES Agar (no adenine):
5 g Yeast extract (DIFCO)
30 g D-Glucose anhydrous (Fisher Scientific)
0.2 g Arginine (Sigma)

0.2 g Histidine (Sigma)
0.2 g Leucine (Sigma)
0.2 g Lysine (Sigma)
0.2 g Uracil (Sigma)
20 g Agar (Sigma)

YES liquid:
5 g Yeast extract (DIFCO)
30 g D-Glucose anhydrous (Fisher Scientific)
0.2 g Arginine (Sigma)
0.2 g Histidine (Sigma)
0.2 g Leucine (Sigma)
0.2 g Lysine (Sigma)
0.2 g Uracil (Sigma)

50X Salts:
53.5 g Magnesium Chloride $6H_2O$
1 g Calcium Chloride $6H_2O$
50 g Potassium Chloride
2 g Di-Sodium Sulphate

1000X Vitamins (100ml):
0.5 g Panthothenic acid
1 g Nicotinic acid
1 g Inositol
1 mg Biotin
Filter sterilized

10,000X Minerals:
5 g Boric acid
4 g $MnSO_4$
4 g $ZnSO_4.7H_2O$
2 g $FeCl_2.6H_2O$
1.6 g Molybdic acid
1 g KI
0.4 g $CuSO_4.5H_2O$
10 g Citric acid
Filter sterilized

Supplement Stocks:
50X Adenine (Sigma) 5 g/L
100X Arginine (Sigma) 10 g/L
100X Histidine (Sigma) 10 g/L
100X Leucine (Sigma) 10 g/L
25X Uracil (Sigma) 2.5 g/L

Malt Extract plates (ME) per liter:	20 g Agar (OXOID) 30 g Malt extract (OXOID) 250 mg Adenine (Sigma) 250 mg Arginine (Sigma) 250 mg Histidine (Sigma) 250 mg Leucine (Sigma) 250 mg Uracil (Sigma)
5-FOA plates:	PMG or YES-Agar 1 g/L FOA (Melford laboratories), added to melted agar below 60°C
TBZ plates:	YES-Agar TBZ (stock 10 mg/ml in DMSO) to 10 μg/ml, added to melted agar below 60°C
Phloxin B plates:	PMG or YES-Agar 2.5 μg/ml Phloxin B

2.2 FISSION YEAST PROTOCOLS

Media and growth

Haploid strains of *Schizosaccharomyces pombe* grow with the following generation times.

Medium	Temperature °C	Generation Time
YES	25	3 h
	32	2 h 10 min
	36	2 h
Minimal	25	4 h
	32	2 h 30 min
	36	2 h 20 min

Temperature sensitive strains were grown at 25°C (permissive temperature) and 36°C (restrictive temperature). Wild type strains were generally grown at 32°C.

Cell culture

For physiological experiments cells were maintained in mid-exponential growth i.e. 2 x 10^6

to 1 x 10^7 cells/ml. To generate cultures in mid-exponential growth, a loop of freshly patched strain was used to inoculate 10 ml media and incubated for 1 - 2 days at the appropriate temperature until cells are in early stationary phase. This pre-culture was used to inoculate a larger culture, taking into consideration generation times.

Flask size should be selected according to culture volume required: 25 ml flask for up to 10 ml culture, 100 ml flask for up to 50 ml, 250 ml for up to 125 ml cultures and 500 ml for up to 250 ml cultures.

Cell counting

Cells were counted using a haemocytometer. A haemocytometer is a specialized microscope slide on which two grids have been engraved in a central region that is 0.1 mm lower than the rest of the slide. Each grid comprises 25 large squares, each containing 16 smaller squares. A coverslip is applied to the slide, and 10 µl of cell culture is pipetted under the coverslip. Multiplying the total number of cells in the 25 large squares by 10^4 gives the number of cells/ml.

Serial dilution assay

To assess the growth of various mutant strains on different media or at different temperatures, cells from a fresh plate were serially diluted 10 fold in sterile dH_2O in a 96 well microtiter plate. Cells were then spotted onto appropriate media using sterilized metal 'hedgehog' and plates were incubated at the desired temperature until colonies appeared.

Auxotrophy

The auxotrophic markers most commonly used in *S. pombe* require uracil, adenine, arginine, leucine, lysine and histidine. Amino acids are used at concentration of 100 µg/ml (4 ml of 10 mg/ml stock solution per 400 ml medium). To test for auxotrophy the strain is grown up to single colonies on YES and then replica plated to minimal medium with and without the appropriate supplement. The plates are incubated for 1 – 2 days and then examined for growth under these conditions.

2.3 MOLECULAR GENETICS

Transformation

50 ml of culture, grown to a density of 1 x 10^7/ml in YES was required for each

transformation. Cells were harvested by spinning at 3000 rpm for 5 minutes at 20°C.

- **Lithium acetate**

Cells were washed once in 10 ml 0.1 M LiAc pH 4.95 and then resuspended in 10 ml 0.1 M LiAc pH 4.95 and incubated at 25/32°C for 30 minutes. Cells were then resuspended at 10^9/ml in 0.1 M LiAc pH 4.95. 150 µl of cell suspension was mixed with 1 µg DNA and 370 µl PEG 3350 (50% solution dissolved in TE pH 8) and then incubated for a further 30 minutes at 25/32 °C. Cells were then heat-shocked at 42°C for 20 minutes. Cells were then resuspended in selective liquid media for at least 3 hours before plated on appropriate selective media.

- **Electroporation**

Cells were washed three times with 10 ml ice-cold 1.2 M sorbitol and after final wash the pellet was resuspended in 500 µl 1.2 M sorbitol. 200 µl of cell suspension was added to chilled transformation cuvette containing 100 ng of DNA (plasmid) or 10 µg DNA (linearised fragments for integration) to be transformed and mixed. Cuvette was placed in slot of electroporator (Biorad) and was pulsed briefly (electroporator settings were 1.5 kV, 200 ohms, 25 µF). 500 µl of 1.2 M sorbitol was then added to the cuvette and mixed gently. Cells were plated as soon as possible onto selective media and transformants appeared after 4 – 6 days at 25 °C. When transforming DNA fragments designed to replace the *ura4*$^+$ gene, 5 – 10 µg of DNA was co-transformed with 100 ng of LEU2$^+$ plasmid and transformants were first selected on –LEU plates, before replica-plating twice to –LEU plates containing FOA.

Expression plasmids using inducible promoters

pREP plasmids containing the thiamine repressible promoter were used for over-expression analysis. These vectors utilize the thiamine responsive promoter of the *nmt1*$^+$ gene. Expression of the gene of interest is repressed in the presence of thiamine and is induced on removal of thiamine from the medium, requiring approximately 16 hours for full induction (Maundrell et al., 1993). The *nmt1* promoter sequence has been mutated to different degrees to give lower levels of expression: pREP3X contains the strongest promoter, pREP41X has a 15 fold lower induced level then pREP3X and pREP81X has an 80 fold lower induced level than pREP3X (Basi et al., 1993).

For tight and rapid regulation protein induction, the *inv1* promoter was utilized. The *inv1*$^+$ gene encodes invertase, an enzyme responsible for sucrose metabolism that is regulated transcriptionally by glucose de-repression in *S. pombe* (Tanaka et al., 1998). The *inv1*$^+$ gene is expressed in the absence of glucose as the derepression, which is normally exerted by the zinc transcription factor Scr1, is released. The *inv1* promoter is rapidly induced on shift

from glucose to sucrose-based culture medium and genes that are regulated by the *inv1* promoter are fully induced within 1 hour of the shift.

For experiments using the *inv* promoter, cells were cultured overnight in PMG supplemented with 10% w/v glucose. For protein induction, cells were centrifuged, washed once in dH_2O and cultured in PMG supplemented with 4% w/v sucrose for 60 minutes before harvesting.

Recovering plasmids from *S. pombe*

To recover plasmids from *S. pombe* cells transformed with the Shimoda genomic library, single colonies were first cultured for a 2 - 3 days under selective conditions (supplemented PMG –LEU) to a density of 1×10^7/ml. Cells were harvested by centrifugation at 3000 rpm for 5 minutes. Pellet was resuspended in 0.5 ml of SP1 buffer containing 1 mg/ml Zymolyase 100-T and incubated at 37°C for 1 hour. Cells were then centrifuged and pellet was resuspended in lysis buffer from Qiagen miniprep kit. Protocol as per miniprep was then followed. 100 µl of DH5α competent cells were transformed with 20 µl of recovered miniprep plasmid and plated on selective LB plates (LB + 30 µg/ml Ampicillin).

SP1 buffer: 1.2 M sorbitol, 50 mM sodium citrate, 50 mM $Na_2HPO_4.7H_2O$, 40 mM EDTA, pH to 5.6 with orthophosphoric acid.

Genetic crosses

Crosses were carried out on nitrogen starved malt extract (ME) medium. To cross two strains, a loopful of a freshly growing h+ strain and a loopful of freshly growing h- strain were mixed together on an ME plate. The cross was then incubated at 25°C to allow conjugation. Fully formed four spore asci are visible after 2 - 3 days incubation at 25°C.

- **Random spore analysis**

A 2 - 3 day old cross was checked for the presence of asci by light microscopy. A loopful of mating mixture was resuspended in 300 µl of 1 in 100 dilution of glusulase and incubated overnight at 25°C. Glusulase is a crude snail gut enzyme that breaks down the vegetative cells and the ascus wall. Between 200 - 1000 spores were then plated on YES agar or selective media at appropriate temperature until colonies are formed.

- **Tetrad dissection**

A fresh 1 - 2 day old cross was used for tetrad analysis as the ascus wall has not yet started to break down at this stage. Asci were placed in a line about 6 mm apart using a Singer Tetrad dissecting microscope on YES agar plate. Asci were then left to breakdown at 18 -

25°C overnight. Each ascus was then micro-manipulated to give a line of four isolated spores and allowed to germinate and form colonies at the appropriate temperature. When colonies appeared, the plate was replica-plated on various media to determine the genotype of cells.

Linkage analysis by crossing

Two mutant strains of interest were crossed; asci were treated with glusulase and plated on agar medium as described. The presence of wild type progeny in a cross between two temperature sensitive strains indicates that the two mutations under observation are in different genes, as recombination has taken place. In this case, Mendelian segregation will result in 25% of spores being wild type, assuming the genes are unlinked. If no wild type progeny are produced, linkage is suspected.

Construction of double mutants

Tetrad dissection was carried out on the progeny of a cross between two temperature sensitive strains. Three types of tetrad are normally produced: the parental ditype (PD) if there is no recombination, the tetratype (TT) if there is a single crossing-over (i.e. two recombinants per ascus) or the non-parental ditype if there is a double crossing-over (i.e. all recombinants produced). The progeny of a cross between two ts strains were grown on YES at 25°C and replica-plated onto YES + Phloxin B and grown at 36 °C. Where non-parental ditypes were produced, two spores were wild type and two spores were ts double mutants.

Yeast-2-hybrid assay

- **Budding yeast Drop Out Media**

For 500 mls: 0.61 g Drop Out CSM (Complete Supplement Mixture) -ade-his-leu-trp
50 mls YNB 10X (33.5g in 500 ml)
1 g Glucose
10 g Agar
0.5 ml NaOH

For additional amino acids (100X stock): histidine 6 mg/ml, tryptophan 8 mg/ml, ade 6 mg/ml, leucine 8 mg/ml

- **Plasmids**

Sim3, CENP-A^{Cnp1} and H3.1 were cloned into pACT2 GAL4 Activation Domain vector (GenBank Accession no. U29899) and into pAS2-1 GAL4 DNA-Binding Domain vector (BD

Biosciences/Clontech).

- ***Saccharomyces cerevisiae* transformation protocol**

50 ml cell culture was grown overnight at 30°C in YPD media and spun for 3 minutes at 1500 rpm to pellet cells. Cells were washed with 1 ml LiOAc mix, transferred to a microfuge tube and pellet was finally suspended in 500 µl LiOAc mix. 15 µl of carrier DNA (10 mg/ml salmon sperm ssDNA) and 1 µg of DNA (pACT2 and pAS2-1 plasmids) was added to 100 µl of cells and mixed. 700 µl of PEG mix was added to the tube and incubated at 30°C for 30 minutes. Cells were heat-shocked for 15 minutes at 42°C, spun down in a microfuge and resuspended in 200 µl YPD before plating on selective media (-tryp, -leu). When colonies appeared, cells were spotted onto media (-tryp, -leu, -his or –tryp, -leu,-ade) to test for positive and negative interactions.

LiOAc mix: 100 mM LiOAc, 10 mM Tris pH 7.4, 1 mM EDTA
PEG mix: 40% PEG (2800); 100 mM LiOAc, 10 mM Tris pH 7.4; 1 mM EDTA

2.4 DNA AND RNA TECHNIQUES

Preparation of genomic DNA

5 ml of stationary phase culture was pelleted at maximum speed in a benchtop centrifuge. The pellet was resuspended in 250 ml SP1 buffer containing 0.4 mg/ml Zymolyase-100T and incubated at 37°C for 1 hour. Spheroplasted cells were pelleted at 8,000 rpm in an eppendorf centrifuge for 15 seconds. The pellet was resuspended in 500 µl TE and 50 µl 10% SDS was added, followed by vortexing and the addition of 165 µl 5 M KOAc. Samples were incubated on ice for 30 minutes, and spun in a microfuge for 10 minutes. The supernatant was then added to 750 µl isopropanol, placed on dry ice for 10 minutes. Samples were spun for 10 minutes in a microfuge and pellet was allowed to dry. Pellet was then resuspended in 300 µl TE containing 10 µg/ml RNase and incubated for 1 h and 30 minutes at 37°C. DNA was then extracted by phenol/chloroform and precipitated with ethanol and 1/10 volume of 3 M NaOAc. Genomic DNA was then resuspended in 20 µl TE.

Rapid genomic DNA preparation for PCR (SPZ)

A small amount of cells was picked from a fresh plate using a sterile cocktail stick and were resuspended in a microfuge tube containing 20 µl of SPZ buffer + 0.5 µl Zymolyase 100-T (10 mg/ml). After mixing, tubes were incubated at 37 °C for 20 minutes. 200 µl of sterile dH_2O was then added, the tubes were vortexed and 2 µl of the mixture was used in a 20 µl PCR reaction.

SPZ buffer: 1.2 M Sorbitol; 100 mM sodium phosphate, pH 7.4; 2.5 mg/ml Zymolyase 100-T. Stored at 20°C.

Preparation of total *S. pombe* RNA

A 10 ml culture of cells was grown in YES or appropriate media to a density of 1 x 10^7/ml. Cultures were pelleted by centrifugation, washed with TE and transferred to microfuge tubes prior to being resuspended in 300 µl RNA extraction buffer. 300 µl glass beads (Sigma) were added followed by 300 µl phenol/chloroform pH 4.7 (Sigma). The microfuge tubes were shaken at high speed on a multi-head vortexer for 30 minutes at 4°C to lyse the cells, followed by centrifugation at 10,000 rpm for 5 minutes and removal of supernatant. The supernatant was extracted with phenol chloroform and then with chloroform. The RNA was precipitated with 3 volumes of cold 100% ethanol and centrifuged at 10,000 rpm for 15 minutes at 4°C. The pellet was air-dried and resuspended in 20 µl dH_2O if used for cDNA synthesis or 50% formamide (Sigma) if used for Northern analysis.

Preparation of RNA for microarray analysis (protocol from K. Ekwall laboratory)

100 ml of cells grown overnight at 25 °C in YES were shifted to 36 °C for 6 hours to mid-exponential phase (1 x 10^7/ml). Cells were harvested and frozen in liquid nitrogen. Pellets were thawed and resuspended in 2 ml TES solution. 500 µl of pellet slurry was immediately added to pre-prepared tube containing 500µl acid phenol. Tubes were vortexed for 10 seconds and incubated for 30 minutes at 65°C on a heat block in hood and were vortexed occasionally. RNA was then acid phenol and chloroform extracted and precipitated with 3M sodium acetate, pH 5.3 and 100% ice-cold ethanol on dry ice. RNA was pelleted and resuspended in 100 µl DEPC treated H_2O. RNA was cleaned up using the RNeasy Mini Protocol (Qiagen) according to manufacturer's instructions. RNA concentration was determined using the nanodrop spectrophotometer. 25 µg of RNA in a volume of less than 20 µl was required for each sample array.

Northern blotting

Total RNA samples were diluted in 3 volumes of sample loading buffer, vortexed and denatured for 10 minutes at 65°C, before cooling on ice for 5 – 10 minutes. Samples were loaded on an agarose-formaldehyde gel and gel was run in the fume hood at 80 V for 4 hours.

Agarose-Formaldehyde gel (for 200 ml): 2 g agarose, 147.6 ml dH_2O, 20 ml 10X HEPES pH 7.8, 32.4 ml Formaldehyde 37% (Sigma)

Running buffer: 1X HEPES pH 7.8

Sample loading Buffer (for 1 ml): 100 µl 10X HEPES pH 7.8, 500 µl Formamide, 160 µl Formaldehyde 37 %, 170 µl 50 % glycerol, 5 µl ethidium bromide, 65 µl dH_2O.

When blue dye reached two-thirds the way down the gel, the gel was under UV photographed next to a fluorescent ruler. The gel was soaked in 5 gel volumes of 2X SCC for 20 minutes. Membrane (Hybond) was first soaked briefly in dH_2O and then in 10X SCC for 10 minutes. In a tray containing 20X SCC, the following stack was assembled: a large gel tray upside-down, 3 large pieces of 3 MM paper soaking in the 20X SCC, 3 gel-sized pieces of 3 MM, the gel upside down, Hybond-NX (Amersham Biosciences) membrane, a large stack of paper towels and a weight of approximately 500 g on top. Care was taken while stacking papers and gel that no air bubbles were formed. The exposed parts of the stack were wrapped in Parafilm to prevent evaporation and the transfer was left overnight.

After disassembly, the location of the wells was marked on the membrane. The membrane was placed on some 3 MM paper and RNA was cross-linked to the membrane using an Autocrosslink Stratalinker (2 times 1200 joules/cm^3). The membrane was stored at room temperature until required.

20X SCC: 3 M NaCl; 0.3 M Citrate tri-sodium

25 ng of cleaned (Qiagen) DNA fragment to be used as probe was diluted to 13 µl with dH_2O, boiled for 10 minutes and put on ice. 4 µl of High Prime (Roche) and 5 µl of α ^{32}P-dCTP were added to the denatured DNA and incubated for 30 minutes at 37°C. The probe was then cleaned using a column, kept on ice and boiled for 5 minutes before use.

Membranes were pre-hybridized in hybridization buffer for 2 hours at 68°C in a hybridization bottle in a roller oven. The probe was boiled for 5 minutes and added immediately to the bottle. The membrane was incubated overnight at 55°C. The membrane was washed once with wash buffer 1 and twice with wash buffer 2, each for 10 minutes at 68°C. The membrane was exposed to a phosphorous screen for 1 – 3 hours before data collection using a Storm phosphor-imager.

Pre-hybridization/Hybridization solution: 0.5 M Na phosphate pH 7.2; 7 % SDS; 1 mM EDTA

Wash solution 1: 1X SCC; 1 % SDS

Wash solution 2: 0.5X SCC; 1 % SDS

2.5 PROTEIN TECHNIQUES

Total protein extraction from fission yeast

A 10 ml culture was grown to log phase in YES or minimal medium and cells were harvested by spinning in a benchtop centrifuge at 3000 rpm for 2 minutes. Pellet was resuspended in 1 ml PEMS and transferred to a microfuge tube. Pellet was then resuspended at 10^8 cells/ml in PEMS containing Zymolyase-100T at 0.4 mg/ml and incubated at 37°C for 20 minutes. Spheroplasted cells were then washed in PEMS and resuspended at 5 x 10^7 cells per 100 µl 2X SB (containing freshly added PMSF). Samples were vortexed vigorously and boiled for 5 minutes at 95°C on a heat block. Samples were spun briefly to pellet cellular debris before loading on gel (10 µl / 5 x 10^6 cells per lane) or freezing at – 20°C.

2X Sample Buffer: 2% SDS; 50 mM Tris-HCl, pH 6.8; 2 mM EDTA; 10% glycerol; 0.03% Bromophenol Blue; 2% β-mercaptoethanol

SDS-PAGE (Laemmli, 1970)

Proteins were separated on 1 mm thick discontinuous SDS-PAGE (Sodium dodecyl sulphate – polyacrylamide gel electrophoresis) with the Hoefer minigel apparatus. The percentage of resolving gel was selected to allow optimum separation of proteins within the size range required.

Resolving gel (for 10 ml):

10%: 3.3 ml 30% acrylamide/bis mix (Sigma); 4 ml dH_2O; 2.5 ml 1.5 M Tris-HCl pH 8.8; 100 µl 10% SDS; 100 µl 10% Ammonium persulphate; 10 µl TEMED

12%: 4 ml 30% acrylamide/bis mix (Sigma); 3.25 ml dH_2O; 2.5 ml 1.5 M Tris-HCl pH 8.8; 100 µl 10% SDS; 100 µl 10% Ammonium persulphate; 10 µl TEMED

15%: 5 ml 30% acrylamide/bis mix (Sigma); 2.25 ml dH_2O; 2.5 ml 1.5 M Tris-HCl pH 8.8; 100 µl 10% SDS; 100 µl 10% Ammonium persulphate; 10 µl TEMED

5% Stacking gel (for 100ml, 2 ml per gel):

17 ml 30% acrylamide/bis mix (Sigma); 12.5 ml 1 M Tris-HCl pH 6.8; 1 ml 10% SDS; 69.5 ml dH_2O

10 µl 10% Ammonium persulphate and 4 µl TEMED were added to 2 ml of stacking gel mix before pouring.

Resolving gel was poured first, followed by stacking gel into which combs were inserted. Gels were run in 1X running buffer at 180V (constant voltage) for approximately 40 minutes. Gels were stained using SimplyBlue SafeStain to reveal protein according to manufacturer's instructions.

5X Running Buffer (for 1 liter): 30 g Tris Base; 144 g Glycine; 5 g SDS

Western analysis

Proteins were transferred on Protran nitrocellulose (Schleicher & Schuell) using a Hoefer semi-dry electroblotter. The membrane floated on dH_2O, soaked in blotting buffer and then placed on top of 6 pieces of 3 MM paper of the gel size. The SDS gel was placed on top of the membrane followed by 6 more pieces of 3 MM paper soaked in blotting buffer. As each layer was added, bubbles were rolled out using a glass tube. Transfer was carried out at the constant amperage of 65 mA for 2 hours. The membrane was washed in dH_2O, followed by staining with Ponceau solution (Sigma) to verify protein transfer. The membrane was washed in PBS, followed by incubation in blocking buffer for 1 hour at room temperature with agitation. The membrane was then placed in a sealed bag and incubated with the primary antibody of interest in PBS – 0.1% Tween-20 overnight at 4°C with agitation. The membrane was washed 3 times each for 15 minutes in PBS – 0.1% Tween-20 and then incubated with appropriate HRP-conjugated secondary antibody of interest in blocking buffer for 1 hour at room temperature. The blot was again washed three times in PBS – 0.1% Tween-20, each for 15 minutes, followed by a final quick wash in PBS. Proteins were revealed using the Enhanced Chemi-Luminescence kit (Amersham) following the manufacturer's instructions. The blot was exposed to Kodak Bio-Max Light film for 10 seconds up to 1 hour.

Blotting buffer: 20 ml 5X SDS running buffer; 60 ml dH_20; 20 ml methanol
Blocking buffer: 5% Marvel dried non-fat milk; 0.1% PBS-Tween

Expression of recombinant fusion proteins in *E.coli*

Glutathione-S-transferase (GST)-tagged fusion proteins were purified under native conditions. BL21 codon plus cells were grown at 37°C to log phase. Fusion protein production was induced by addition of IPTG to 100 μM and cells were grown for a further 3 - 5 hours. All subsequent stages are performed on ice/at 4°C. Cells were harvested by spinning at 5,000 rpm for 20 minutes in a Beckman centrifuge. Cells were then washed in ice-cold PBS and were snap frozen in liquid Nitrogen. Pellet was thawed on ice, re-suspended in ice-cold PBS + 1% Triton X-100, sonicated and centrifuged for a further 10

minutes at 5,000 rpm. A 50:50 slurry of pre-swollen glutathione agarose (Sigma) in PBS was added to recovered supernatant and rotated gently for 30 minutes. Beads were washed 3 times in ice-cold PBS + 1% TritonX-100. Fusion protein was eluted in freshly prepared 5 mM glutathione in 50 mM Tris-HCl pH 8, 250 mM KCl for 5 minutes. Samples were dialysed into PBS + 20% glycerol overnight at 4°C.

Cleavage of recombinant fusion proteins

GST was cleaved from GST-Sim3 fusion protein using THROMBIN CleanCleave kit (SIGMA). 10 µg of GST-Sim3 was incubated with a 50:50 suspension of thrombin-agarose beads in 1X cleavage buffer (500 mM Tris-HCL pH 8, 100 mM $CaCl_2$) for 1 - 2 hours at room temperature.

Pulldown of *in vitro* transcribed/translated proteins with GST-fusion proteins

^{35}S-labelled proteins were produced using the TNT T7 Quick for PCR DNA kit (Promega) according to manufacturer's instructions. 40 µl of TNT Quick master-mix, 4 µl ^{35}S-Methionine and 500 ng of PCR DNA fragment containing a T7 promoter were mixed and incubated at 30°C for 60 - 90 minutes.

For each reaction, 4 µg of fusion protein and 10 µl of ^{35}S-labelled protein were incubated on ice for 30 minutes in binding buffer. 20 µl of a 50:50 slurry of glutathione agarose beads in binding buffer was added and samples were rotated for 1 hour at 4°C. Beads were washed 4 times in ice-cold binding buffer. On final wash, supernatant was removed and beads were boiled in 20 µl 2X sample buffer and loaded on gel. Gel was fixed (25 ml isopropanol, 65 ml dH_2O, 10 ml acetic acid) for 30 minutes at room temperature, then soaked in Amplify for 45 minutes and then dried on a gel dryer. Gel was exposed to Hyperfilm overnight at -80°C and developed.

Binding buffer: 50 mM HEPES pH 7.6, 75 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, 1 mM PMSF, Protease inhibitors.

Chromatin immunoprecipitation (ChIP)

50 ml of exponentially growing cells (5×10^6; 2.5×10^8 cells were required per ChIP) were fixed for 5 to 30 minutes (depending on protein to be ChIP'd and temperature of culture) with 3% parafolmaldehyde dissolved in YES (+ 10 M NaOH to neutralise). Fixation was stopped by the addition of 2.5 M glycine (20X) to cultures for 5 minutes at room temperature with agitation. Cells were then washed twice in 20 ml of ice-cold PBS, resuspended in 1 ml PEMS (100 mM Pipes, pH 6.9; 1 mM EDTA; 1 mM Mg_2SO_4, 1.2 M

Sorbitol) and transferred to a microfuge tube. Pellet was then resupended at 10^8 cells/ml in PEMS containing 0.4 mg/ml Zymolyase 100-T and incubated at 37 °C for 20 - 30 minutes. Cells were then washed twice in PEMS (pellets may be frozen at –20 °C in 'one ChIP' size aliquots at this point). Pellet was then resuspended in 300 µl of lysis buffer containing protease inhibitor cocktail (100X Sigma) and 2 mM PMSF. Lysates were then sonicated using a water bath sonicator for 4 x 5 minutes. This should result in shearing the chromatin to approximately 500 – 1000 bp. After sonication, lysate was adjusted to a total volume of 400 µl. Tubes were spun for 5 minutes at 13,000 rpm at 4°C, supernatant was removed to new tube and spun for 15 minutes at 13,000 at 4°C to remove debris. Cleared lysate was pre-cleared by adding 25 µl of Protein A or Protein G Agarose and were incubated with gentle rocking for 1-2 hours at 4°C. Protein A/G Agarose was washed 3 times in lysis buffer and made into a 50:50 v/v suspension of beads in lysis buffer. Protein A Agarose is used for rabbit antibodies and Protein G Agarose is used for sheep antibodies. After pre-clearing, beads were spun at 8,000 rpm for 2 minutes at 4°C and supernatant was transferred to a new tube using a duckbilled pipette (Sorenson BioScience Inc.). 40 µl of this pre-cleared lysate was frozen as 'crude input' sample. The appropriate amount of antibody was added to the remaining lysate for 4 hours to overnight at 4°C:

PEM: 100 mM Pipes, pH 6.9; 1 mM EDTA; 1 mM Mg_2SO_4
PEMS: PEM containing 1.2 M Sorbitol

Beads were then spun at 8,000 rpm and washed for 10 minutes at 4°C with rocking with 1 ml of each of the following buffers: lysis buffer, lysis buffer + 500 mM NaCl, wash buffer, TE pH 8. After the washes, 250 µl TES was added to beads, 210 µl TES was added to the 'crude input' sample also and tubes were incubated overnight at 65°C to reverse the cross-linking. 30 µl of 10 mg/ml Proteinase K (Roche) and 450 µl TE was then added and tubes were incubated at 37°C for 2 hours. Samples were then phenol/chloroform and chloroform extracted and DNA was precipitated with 1/10 volume 3 M NaOAc pH 5.5, 2.5 volumes of ice-cold 100% ethanol and 1.5 µl of 10 mg/ml Glycogen was added to help view pellet. Samples were mixed thoroughly by vortexing and incubated on dry ice for 1 hour. DNA was recovered by centrifugation at 4°C for 30 minutes at maximum speed. The pellet was dried under the fume hood for 15 to 20 minutes. ChIP (IP) DNA was resuspended in 30 µl and crude input DNA (T) in 300 µl TE. 2 µl of DNA were used in 20 µl PCR reactions with appropriate multiplex primer sets with added Mg^{2+}.

The 'ura' program was used for all the PCR of ChIP DNA described in this thesis: 94°C 4 min; (94°C 30 seconds; 55°C 30 seconds; 72°C 1 minute) 30 times; 72°C 5 minutes.

Lysis buffer: 50 mM HEPES-KOH, pH 7.5; 140 mM NaCl; 1 mM EDTA, 1% Triton X-100; 0.1% (w/v) sodium deoxycholate

Wash buffer: 10 mM Tris-HCl, pH 8; 0.25 M LiCl; 0.5% NP-40; 0.5% (w/v) sodium deoxycholate; 1 mM EDTA
TE: 10 mM Tris-HCl, pH 8; 1 mM EDTA
TES: 50 mM Tris-HCl pH 8; 10 mM EDTA; 1% SDS

For anti-H3K9me2 ChIP (mAb 5.1.1, a gift from Takeshi Urano lab), the standard ChIP protocol was used with the following modifications. Cells were fixed with 1% formaldehyde for 15 minutes (2.5×10^8 cells per ChIP). On washes with PBS, cells were transferred to a round-bottomed screw-capped tube. After the addition of lysis buffer, 500 µl of small glass beads were added and cells were bead-beaten on ice for 2 times 2 minutes. To isolate supernatant from cellular debris, the bottom of the screw-capped tube was pierced with a needle and tube was placed inside a 15 ml falcon tube containing a microfuge tube for collection. Tubes were centrifuged at low speed 1000 rpm for 1 minute and lysate was collected in microfuge tube. Samples were then sonicated 3 times for 5 minutes in a water bath sonicator. The H3K9me2 antibody was pre-coupled to Protein G Agarose beads for 4 hours at 4°C before use (1 µl antibody + 25 µl beads (50:50 slurry) + 500 µl cold antibody binding buffer). 25 µl of pre-coupled antibody was subsequently added to each sample with slow rotation overnight at 4°C.

Antibody binding buffer: 0.5% NP-40; 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$

Immunoprecipitation

100 ml of cell culture was grown to the concentration of 5×10^6 cells/ml. Cells were spun in a benchtop centrifuge for 2 minutes at 3,000 rpm and washed once with dH_2O. Cells were transferred to a 2 ml flat-bottomed screw-cap tube, centrifuged and pellets frozen in liquid N_2. Pellets were resuspended in 500 µl of ice-cold Lysis buffer containing protease inhibitors (100X Sigma) and 1 mM PMSF (100 mM stock in methanol at –20°C). 500 µl glass beads (acid-washed Sigma) were added and cells were lysed in bead-beater two times, each for 2 minutes, with incubation on ice between beatings. Cells were spun briefly to pellet beads/debris and transferred to new tube. DTT was added to 0.5 mM and lysate was spun three times for 5 minutes, transferring the supernatant to a fresh tube. The lysate was pre-cleared for 1 hour at 4°C with rotation with 25 µl Protein A or Protein G Agarose (Roche) as appropriate that has been washed twice in Lysis buffer and resuspended as a 50:50 v/v beads in buffer. The pre-cleared lysate was transferred to a fresh tube and 20 µl was kept as whole cell extracts (WCE) and stored at –20°C. Desired antibody was added to remaining lysate and samples were incubated with gentle rotation at 4°C for 1 hour. 25 µl of appropriate Protein A/G Agarose was added and immunoprecipitations were incubated with gentle rotation at 4°C overnight. Beads were washed 3 times with 500 µl of ice-cold

Lysis buffer and at the last wash transferred to a new tube. Beads were washed a further two times with PBS and the supernatant was removed using duckbilled tips. 20 µl of 2X sample buffer were added to IP and WCE samples and tubes were heated at 65°C for 10 minutes. 10 – 20 µl of material was loaded on a SDS-PAGE gel of appropriate percentage and followed by Western blot analysis.

Lysis buffer: 50 mM Hepes, pH 7.6; 75 mM KCl; 1 mM $MgCl_2$; 1 mM EGTA; 0.1% TritonX-100

Antibody Affinity purification

- **Nitrocellulose method**

Cleaved Sim3 protein was blotted to nitrocellulose, Ponceau stained and cut from the membrane using a sharp scalpel. The membrane strips were minced finely (2mm squares), washed with PBS 0.1% Na azide, and incubated with diluted sera (1:5 in PBS 0.1% Na azide) overnight at 4°C. 1X used sera was then removed to a fresh eppendorf and strips were washed twice with 1 ml PBST for 30 minutes at room temperature. Antibodies were eluted by the addition of 200 µl of elution buffer (200 mM glycine pH 2.8, 0.1% BSA, filter sterilised) for 3 hours at room temperature. Supernatant was removed to a new tube followed by two quick elutions of 50 µl of elution buffer. To neutralize 14 µl of 1 M Tris base (not pHd) was added to 300 µl of eluted antibody and antibody was then dialysed against PBS 0.1% Na azide overnight at 4°C.

- **Column chromatography (using SulfoLink coupling gel)**

For affinity purification of anti-NASP antibodies, synthesized N terminal and C terminal peptides (PickCell laboratories) were first covalently bound in a column to SulfoLink Coupling Gel (Pierce Biotechnology). Gel slurry was firstly added to column and washed 4 times in coupling buffer (50 mM Tris, 5 mM EDTA, pH 8.5). Peptide was dissolved in sample preparation buffer (0.1 M $NaHPO_4$, 5 mM EDTA, pH 6) at a concentration of 10mg/ml and incubated with gel for 15 minutes at room temperature with rocking. Column was then washed 3x coupling buffer, 6x 1 M NaCl and stored in PBS 0.05% Na azide at 4°C. Column was then equilibrated using 10 mM Tris pH 7.5 and washed in 100 mM glycine pH 8.8 and 100 mM triethylamine pH 11.5 (check each wash with pH paper). Serum was centrifuged for 10 minutes at 15,000 rpm. Diluted serum (1:10 in Tris pH 7.5) was passed over column three times. Following antibody binding, column was washed with 10 mM Tris pH 7.5, 0.5 M NaCl, 0.01% Tween-20. Antibodies were eluted under acidic conditions with 5 ml 100mM glycine pH 2.5 (10 x 500 µl aliquots each into 100 µl Tris pH 8 to neutralise). Column was then washed with 10 mM Tris pH8.8. Antibodies were eluted under alkaline conditions with 100 mM triethylamine pH 11.5 (10 x 500 µl aliquots each into 100 µl Tris pH 8 to neutralise). Fractions were tested for presence of protein using Amido

Black reagent (BioRad) and fractions were antibody eluted were pooled and dialysed into PBS 0.01% Na azide overnight at 4°C.

2.6 MICROSCOPY

Live analysis of Sim3-GFP and GFP-CENP-A^{Cnp1} expressing strains

Cells were grown to log phase, pelleted and re-suspended in a small volume of culture medium. 1% low melting point agarose (Gibco) was boiled and cooled to 37°C, ready for use. 4 µl of cells and 6 µl of agarose were mixed on a microscope glass slide and a glass coverslip was quickly applied on top and pushed down firmly.

Immunostaining

20 ml of a cell culture was grown to a concentration of 5 x 10^6 cells/ml. Cells were fixed by the addition of 3.7% paraformaldehyde dissolved in culture medium (a 10X stock was dissolved at 65°C and cooled down to room temperature) and the culture was shaken at room temperature for the appropriate time. Cells were spun in a benchtop centrifuge at 18°C, washed once with 10 ml PEM (100 mM Pipes, pH 6.9; 1 mM EDTA; 1 mM Mg_2SO_4), transferred to a microfuge tube and washed twice with PEMS. Cells were then incubated at 37°C for 90 minutes in PEMS containing 1 mg/ml Zymolyase 100-T (ICN) at a concentration of 10^8 cells/ml. After washing in 1 ml PEMS, cells were resuspended in 1 ml PEMS containing 1% Triton-X100 and incubated on the bench for 5 minutes. Cells were then washed once with PEM, resuspended in 500 µl PEMBAL (PEM containing 1% BSA (Sigma); 0.1% Na Azide; 100 mM Lysine hydrochloride (BDH)) and incubated on a rotating wheel for 1 hour at room temperature. Aliquots of cells were then taken to be incubated with the appropriate dilution of primary antibody in 100 µl of PEMBAL overnight at 4°C on a rotating wheel.

After incubation with primary antibody, cells were washed three times with 1 ml PEMBAL incubating for at least 30 minutes for each wash. The required secondary antibody (Molecular Probes ALEXA anti-mouse, anti-sheep or anti-rabbit) conjugated to the desired fluorescent probe (TRITC or FITC) were added at the concentration of 1: 1000 in 100 µl PEMBAL. Tubes were wrapped in foil and incubated for 4 hours at room temperature with rotation or overnight at 4°C. Cells were washed once for 30 minutes in PEMBAL and incubated for 5 minutes in PEM + 0.1% Sodium Azide containing 1 mg/ml DAPI (stock 500X stored at –20°C). Cells were finally spun and resuspended in 20 µl PEM + 0.1% Sodium Azide. 3 µl of cells were spread in a thin layer on a Poly-L-lysine coated glass slide and allowed to dry. 1 drop of mounting medium Vectashield (Vector Laboratories Inc.) was

then applied to the slide and a coverslip was gently lowered at an angle over the slide to prevent the formation of air bubbles. The coverslips were sealed with transparent nail varnish and observed using a Carl Zeiss MicroImaging, Inc. Axioplan 2 IE fluorescence microscope equipped with Chroma 83000 and 86000 filter sets, Prior ProScan filter wheel (Prior Scientific), and Photometrics CoolSnapHQ CCD camera (Roper Scientific). Image acquisition was controlled using Metamorph software (Universal Imaging Corp.).

PEMBAL: PEM containing 1% BSA (Sigma); 0.1% Na Azide; 100 mM Lysine hydrochloride (BDH)

Formaldehyde-Glutaraldehyde method for staining microtubules

For immunolabelling of microtubules, cells were fixed with 3.7% freshly prepared paraformaldehyde, followed by the addition 0.0625% glutaraldehyde one minute later for a total of 10 minutes at room temperature. Cells were washed, spheroplasted and permeabilised with 1% Triton-X100 as described above. Free aldehyde groups resulting from the glutaraldehyde fixation were then reduced by washing the cells three times for 10 minutes with 2 mg/ml sodium borohydride in PEM. Sodium borohydride solution was prepared immediately before use. Cells were then washed three times in PEM (care was taken as the pellet was floaty and difficult to pellet at this point) and blocked with PEMBAL as described above. Aliquots of cells were then resuspended in 100 µl PEMBAL containing TAT anti-α-tubulin mouse monoclonal antibody at dilution 1:15 and incubated overnight at 4°C with rotation.

2.7 BACTERIAL METHODS

Bacterial cells DH5α were used for all the cloning performed in this thesis. Cells were grown at 37°C in LB medium, solid or liquid, supplemented with 30 µg/ml Ampicillin for plasmid selection.

Bacterial media

LB (per liter): 10g Bacto-peptone; 5g Yeast extract; 10g NaCl. Autoclaved.
Antibiotics: Plates contained 30 µg/ml Ampicillin for plasmid selection. Carbenecillin 50 µg/ml, Chloramphenicol 20 µg/ml.

Transformation of competent cells

Bacterial cells DH5α (Invitrogen, subcloning efficiency) were used for all cloning performed

in this thesis. Bacterial cells BL21 codon plus (Stratagene) were used for all recombinant protein production performed in this thesis. An aliquot of frozen competent bacteria was thawed on ice. 50 µl of cells were added to 15 µl ligation mixture or 50ng plasmid DNA and incubated on ice for 30 minutes. Cells were heat-shocked for 90 seconds at 42°C followed by 2 minutes on ice. 200 µl of LB was added and cells were incubated at 37°C for 30 - 45 minutes before spreading on LB-agar plates containing appropriate antibiotic using glass beads.

Plasmid construction

Restriction enzymes were obtained New England BioLabs and were used in the reaction buffer supplied by the manufacturer. Fragments were amplified using Phusion High-Fidelity DNA Polymerase (New England Biolabs). Digests were carried out at 37°C for 2 – 4 hours. Digested fragments were gel-purified using the gel-extraction kit (Qiagen), following the instructions supplied by the manufacturer. Ligations were carried out using T4 DNA-ligase (Roche) in the supplied buffer over night at room temperature.

Plasmid minipreps

2 - 3 ml of LB + Amp bacterial cultures were grown overnight from a single colony. Plasmids were isolated using a miniprep kit (Qiagen) following the instructions provided by the manufacturer.

2.8 *DROSOPHILA MELANOGASTER* S2 CELL PROTOCOLS

S2 cells were grown in plastic flasks at 27°C in Schneider's Drosophila medium (Gibco) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS). Cells expressing CENP-A^{CID}-GFP were selected in media supplemented with 100 µg/ml Hygromycin.

Preparation of dsRNA (adapted from Sharron Vass, Heck laboratory)

Templates for *in vitro* transcription were prepared generated using primers (T7-5-DmSim3 and T7-3-DmSim3 or EB-T7-5 and EB-T7-3) to amplify a 600 bp DNA product of DmNASP or DmEB1 from fly genomic DNA, each with 5' T7 polymerase binding site TTA ATA CGA ACT ATA GGG AGA at both ends. PCR product should be a concentration of 125 ng/µl or greater as the dsRNA reaction requires 1 µg of template in less than 8 µl. To prepare dsRNA reactions were assembled according to supplied protocol of Ambion MEGAscript T7 kit cat.1334 and were incubated for 4 hours at 37°C. Reactions were then ethanol precipitated, spun to removed ethanol, air dried and resuspended in 40 µl of RNAse free

dH_2O. To form doubled stranded RNA, reaction tubes were heated to 65°C for 30 minutes and then placed in a beaker of water also at 65°C and allowed to cool slowly on the bench room temperature to anneal the RNA. Samples were stored at -80°C until required.

RNAi on S2 cells

RNAi on S2 cells was carried out essentially as performed by Clemens et al., 2000, Maiato et al., 2003. Exponentially growing cells were diluted to $1x10^6$ cells/ml in serum free Schneider's medium (Invitrogen). 1 ml of cells was placed in each well of a 6 well 35mm plate (Coring) and 15 μg of dsRNA was added. Another 15 μg of dsRNA was added again after 72 hours to improve efficiency of knockdown. Cells were swirled immediately to mix and incubated for 1 hour at room temperature. After 1 hour, 2mls of Schneider's medium supplemented with 10% FBS (insect qualified) was added to each well. Cells were incubated for 1 to 6 days at 27°C in a humidified incubator.

Immunostaining

Cells were cytospun onto poly-L-lysine coated slides, immediately fixed in 4% para-formaldehyde (PFA) for 3 minutes at room temperature and permeabilised with PBS + 1% Triton X-100 for a further 3 minutes at room temperature. Slides were blocked with PBS + 0.1% Triton X-100 + 3% BSA for 1 hour at room temperature. Antibodies were diluted in PBS + 0.1% Triton X-100 + 0.3% BSA and incubations were performed at room temperature using a humid box. Slides were washed between antibody incubations with PBS + 0.1% Triton X-100. The appropriate fluorescent-conjugated secondary antibody (as described above) was used at a final dilution of 1 in 1000. After antibody staining, cells were treated briefly with DAPI (final concentration 1 μg/ml in PBS). After 30 minutes, slides were washed 3 times in PBS + 0.1% Triton X-100 and were allowed to dry briefly before 1 drop of Vectashield was applied and coverslip was gently lowered over cells and cover slip was sealed with nail varnish.

Total protein extraction

Cells were harvested, counted using a haemocytometer and spun at 2,000 rpm for 4 minutes at room temperature. Medium was removed and cells were washed briefly in 5 ml of PBS. Cells were re-suspended at 5×10^7/ml in 2X sample buffer containing (1 mM PMSF and Protease inhibitors). Samples were sonicated for 2 minutes at full power, boiled for 3 minutes at 95°C and stored at -20°C. 5×10^5 cells were loaded per lane for western blotting.

Production of *Drosophila melanogaster* GST-fusion proteins

DmNASP was amplified from cDNA IMAGE clone LD44305 (MRC geneservice). To clone other fly fusion proteins total RNA was first extracted from S2 cells using RNeasy Mini Protocol (Qiagen) according to manufacturer's instructions. Total cDNA was amplified from by priming total RNA with oligo dT and resulting cDNA was used as template to clone CENP-A^{CID}, H3.3, H4 and H1.

ANTIBODIES USED IN THIS THESIS

Western analysis:

rabbit anti-Sim3 (1:1000) affinity purified 6376, rabbit anti-GFP (1:1000) Molecular Probes, mouse anti-GFP (1:500) BD Living Colours, anti-alpha-tubulin (1:500) (I. Hagan), rabbit anti-Myc Research Diagnostics (1:1000), affinity purified rabbit anti-CENP-A^{Cnp1} (1:100) (M. Yanagida), rabbit anti-NASP N-term (1:1000), rabbit anti-NASP C-term (1:1000), rabbit anti-EB1 (1:5000) (H. Okhura), rabbit anti-H3C AbCam (1:2,500), sheep anti-GFP (K. Hardwick) (1:2000), rabbit anti-Bip1 (A. Pidoux) (1:5000), mouse anti-HA 12C (W. Earnshaw) (1:300).

Immuno-fluorescence:

sheep anti-CENP-A^{Cnp1} serum (1:300), mouse anti-alpha-tubulin (1:15) I. Hagan, rabbit anti-Sad1 (1:40), rabbit anti-NASP N-term (1:100), rabbit anti-NASP C-term (1:100), rabbit anti-GFP Molecular Probes (1:50), rabbit anti-Sim3 (1:40) affinity purified 6376, anti-his-HRP (1:1000).

Chromatin IP:

sheep anti-CENP-A^{Cnp1} (1:30), mouse anti-diMeH3K9 (mAb 5.1.1, a gift from Takeshi Urano lab) (1:400), rabbit anti-H3C AbCam (1:50), rabbit anti-GFP (1:250) Molecular Probes.

Immunoprecipitation:

sheep anti-CENP-A^{Cnp1} (1:40)

STRAINS USED IN THIS THESIS

FY	RELEVANT GENOTYPE
972	*h-*
96	*h- leu1-32 his3-D1 ade6-216*
129	*h- nda3-KM311 leu1-32*
151	*h- cdc25-22 leu1-32*
707	*clr4Δ leu1-32 ade6-210 ura4-DSE otr1R dg/glu(Bam/Spe1) Sph1::ura4*
1180	*h+ ade6-210 leu1-32 ura4-D18 otr1R (SphI): ade6$^+$*
1181	*h- ade6-210 leu1-32 ura4-D18 otr1R (SphI): ade6$^+$*
1645	*h+ ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*
1646	*h- ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*
3027	*h+ cnt1:arg3$^+$ otr2:ura4$^+$ cnt3:ade6$^+$ tel1:his3$^+$ ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*
3033	*h- cnt1:arg3$^+$ otr2:ura4$^+$ cnt3:ade6$^+$ tel1:his3$^+$ ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*
3606	*h- rik1::LEU2$^+$ cnt1:arg3$^+$ otr2:ura4$^+$ cnt3:ade6$^+$ tel1:his3$^+$ ade6-210 arg3-D3 his3-D1 leu1-32 ura4-D18/DS-E*
3917	*h+ pREP42X-EGFP-CENP-A^{Cnp1}::ura4$^+$(MluI) mis6-3HA::LEU2*
4373	*h? sim3-143 ade6-210 ura4-D18 leu1-32 his3-D1 arg3-D4*
4462	*h- sim2-76 cnt1:arg3$^+$ otr2:ura4$^+$ cnt3:ade6$^+$ tel1:his3$^+$ ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*
4468	*h+ sim6-86 cnt1:arg3$^+$ otr2:ura4$^+$ cnt3:ade6$^+$ tel1:his3$^+$ ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*
4472	*h+ sim2-87 cnt1:arg3$^+$ otr2:ura4$^+$ cnt3:ade6$^+$ tel1:his3$^+$ ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*
4516	*h- sim2-109 cnt1:arg3$^+$ otr2:ura4$^+$ cnt3:ade6$^+$ tel1:his3$^+$ ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*
4536	*h+ sim4-193 cnt1:arg3$^+$ otr2:ura4$^+$ cnt3:ade6$^+$ tel1:his3$^+$ ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*
4812	*h+ mal2-GFP-KAN^R ade6-210 leu1-32 ura4-D6*
5496	*h- sim3-205 cnt1:arg3$^+$ otr2:ura4$^+$ cnt3:ade6$^+$ tel1:his3$^+$ ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*
5691	*h/mis6-302 cnt1:arg3$^+$ otr2:ura4$^+$ cnt3:ade6$^+$ tel1:his3$^+$ ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*
5927	*h+ 3Xmyc-CENP-A^{Cnp1} leu1-32 ura4-D18 his3-D1 ade6-210*
6154	*h? sim3-143 cnt1:arg3$^+$ otr2:ura4$^+$ cnt3:ade6$^+$ tel1:his3$^+$ ade6-210 arg3-D3 his3-D1 leu1-32 ura4-D18/DS-E*
6308	*h? sim3-143-GFP-his3$^+$ cnt1:arg3$^+$ ade6-210 ura4-D18 leu1-32 his3-D1 arg3-D4*
6326	*h+ sim3$^+$-GFP-his3$^+$ cnt1:arg3$^+$ ade6-210 ura4-D18 leu1-32 his3-D1 arg3-D4*
6960	*h- cnp1Δ::ura4$^+$ lys1$^+$::cnp1-1 leu1-32*
7166	*h? cos1-86 cnt1:arg3$^+$ otr2:ura4$^+$ cnt3:ade6$^+$ tel1:his3$^+$ ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*
7167	*h? cos1-7 cnt1:arg3$^+$ otr2:ura4$^+$ cnt3:ade6$^+$ tel1:his3$^+$ ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*
7168	*h? cos1-17 cnt1:arg3$^+$ otr2:ura4$^+$ cnt3:ade6$^+$ tel1:his3$^+$ ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*
7169	*h? cos1-22 cnt1:arg3$^+$ otr2:ura4$^+$ cnt3:ade6$^+$ tel1:his3$^+$ ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*
7170	*h? cos1-38 cnt1:arg3$^+$ otr2:ura4$^+$ cnt3:ade6$^+$ tel1:his3$^+$ ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*
7171	*h? cos2-10 cnt1:arg3$^+$ otr2:ura4$^+$ cnt3:ade6$^+$ tel1:his3$^+$ ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*
7172	*h? cos2-26 cnt1:arg3$^+$ otr2:ura4$^+$ cnt3:ade6$^+$ tel1:his3$^+$ ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*
7173	*h? cos2-28 cnt1:arg3$^+$ otr2:ura4$^+$ cnt3:ade6$^+$ tel1:his3$^+$ ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*
7174	*h? cos2-33 cnt1:arg3$^+$ otr2:ura4$^+$ cnt3:ade6$^+$ tel1:his3$^+$ ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*

7175 *h? cos3-24 cnt1:arg3^{+} otr2:ura4^{+} cnt3:ade6^{+} tel1:his3^{+} ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*

7176 *h? cos4-13 cnt1:arg3^{+} otr2:ura4^{+} cnt3:ade6^{+} tel1:his3^{+} ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*

7177 *h? cos5-35 cnt1:arg3^{+} otr2:ura4^{+} cnt3:ade6^{+} tel1:his3^{+} ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*

7178 *h? cos1-7 ade6-210 leu1-32 ura4-D18 otr1R (SphI): ade6^{+}*

7179 *h? cos1-38 ade6-210 leu1-32 ura4-D18 otr1R (SphI): ade6^{+}*

7460 *h? sim3-143 5myc-CENP-A^{Cnp1} leu1-32 ura4-D18 his3-D1 ade6-210*

7461 *h? sim3-205 myc-CENP-A^{Cnp1} leu1-32 ura4-D18 his3-D1 ade6-210*

8481 *h- ura4::pINV-GFP-CENP-A^{Cnp1}leu1-32 his3-D1 ade6-216*

8482 *h- sim3-143 ura4::pINV-GFP-CENP-A^{Cnp1}leu1-32 his3-D1*

8483 *h+ sim3-205 ura4::pINV-GFP-CENP-A^{Cnp1}leu1-32 his3-D1*

8484 *h- sim3-143 ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*

8519 *h- mis6-302 ura4::pINV-GFP-CENP-A^{Cnp1}leu1-32 his3^{-}*

8518 *h+ cdc25-22ura4:: pINV-GFP-CENP-A^{Cnp1}leu1-32*

8524 *h- sim3-205 ade6-210 arg3-D4 his3-D1 leu1-32 ura4-D18/DS-E*

8717 *h+ cdc25-22 sim3-143 ura4:: pINV-GFP-CENP-A^{Cnp1} leu1-32 his3^{-}*

8718 *h+ cdc25-22 sim3-205 ura4::pINV-GFP-CENP-A^{Cnp1} leu1-32 his3^{-}*

PRIMERS USED IN THIS THESIS

NAME	SEQUENCE	DESCRIPTION
mcl1-seq1	TCCTAGATATGCCCATACAG	*mcl1*⁺ sequencing primer
mcl1-seq2	ACTTGATTAGAACCGACCGT	*mcl1*⁺ sequencing primer
mcl1-seq3	ACCGAGCATACATTGCTGGC	*mcl1*⁺ sequencing primer
mcl1-seq4	CGAAGCCTTAGCTGGACGAA	*mcl1*⁺ sequencing primer
mcl1-seq5	AGGCAGAATCAGAAATTTGC	*mcl1*⁺ sequencing primer
mcl1-seq6	CTGCCAGGCGAGTGCCACCAC	*mcl1*⁺ sequencing primer
mcl1-seq7	GAAGCCTTTGACTTCGTCAA	*mcl1*⁺ sequencing primer
mcl1-seq8	AAAGATCATCAAGCTCTTTG	*mcl1*⁺ sequencing primer
mcl1-seq9	CATGATGGATTGGATTCATC	*mcl1*⁺ sequencing primer
mcl1-seq10	GTGTACTTCCAGTATGAACG	*mcl1*⁺ sequencing primer
mcl1-seq11	ATGCTTAGATCATGAAGGTG	*mcl1*⁺ sequencing primer
mcl1-seq12	AGACGAGAATTTCCATCAGA	*mcl1*⁺ sequencing primer
mcl1-seq13	GGAGATCCTTACATCTACGA	*mcl1*⁺ sequencing primer
mcl1-seq14	GTATTACAAGGAATACGAAA	*mcl1*⁺ sequencing primer
mcl1-seq15	AGGAGATGGCGATGTTACGG	*mcl1*⁺ sequencing primer
mcl1-seq16	ATGGCAGGGAATAGGCTAGT	*mcl1*⁺ sequencing primer
mcl1-seq17	TTAGACGTTAGATAGTAGATT	*mcl1*⁺ sequencing primer
mcl1_promo	GCAATTTCTCAATAGTGCTTTC	sequencing primer in *mcl1*⁺ promoter region
198 sim3-5-EcoR1	TACTACGAATTCATGTCTTCTG ATACGAAAACACTG	forward primer for *sim3*⁺ cloning into pGEX-4T-1
199 sim3-3-Xho1	TACTACCTCGAGTTAATCCTTC TTTTTCTTATCTTTAGGACC	reverse primer for *sim3*⁺ cloning into pGEX-4T-1
200 cnp1-5-Nde1-R1	TACTACCATATGGAATTCATGG CAAAGAAATCTTTAATGGC	forward primer for *cnp1*⁺ cloning into pGEX-4T-1
201 cnp1-3-Xho1 -BamH1	TACTACCTCGAGGGATCCTCAA GCACCACGAATCCTCCTGG	reverse primer for *cnp1*⁺ cloning into pGEX-4T-1
263 pGEX-5	TATAGCATGGCCTTTGCAGGG	pGEX forward sequencing primer
264 pGEX-3	CTTACAGACAAGCTGTGACCG	pGEX reverse sequencing primer
183 577-5-Nde1	TACTACCATATGTCTTCTGATA CGAAAACACTG	cloning *sim3*⁺ into pas2-1
184 577-3-BamH1	TACTACGGATCCTTAATCCTTC TTTTTCTTATCTTTAGGACC	cloning *sim3*⁺ into pas2-1
200 cnp1-5-Nde1-R1	TACTACCATATGGAATTCATGG CAAAGAAATCTTTAATGGC	cloning *cnp1*⁺ into pas2-1
201 cnp1-3-Xho1 -BamH1	TACTACCTCGAGGGATCCTC AAGCACCACGAATCCTCCTGG	cloning *cnp1*⁺ into pas2-1
h3.1-5-Nde1	TACTACCATATGGCTCGTACTAA ACAAACAGCT	cloning *hht1*⁺ into pas2-1

h3.1-3-BamH1	TACTACGGATCCTTATGAGCGTT CGCCACGGAG	cloning *hht1*$^+$ into pas2-1 and pACT2
207 sim3-5-Nco1	TACTACGCCATGGGCATGTCTTC TGATACGAAAACACTG	cloning *sim3*$^+$ into pACT2
184 577-3-BamH1	TACTACGGATCCTTAATCCTTCTT TTTCTTATCTTTAGGACC	cloning *sim3*$^+$ into pACT2
208 cnp1-5-Nco1	TACTACGCCATGGGCATGGCAAA GAAATCTTTAATGGC	cloning *cnp1*$^+$ into pACT2
201 cnp1-3-Xho1 -BamH1	TACTACCTCGAGGGATCCTCAA GCACCACGAATCCTCCTGG	cloning *cnp1*$^+$ into pACT2
h3.1-5-Nco1	TACTACGCCATGGGCATGGCTCG TACTAAACAAACAGCT	cloning *hht1*$^+$ into pACT2
187 sim3-seq-1	ATACGCTTCATCTTACTCCC	*sim3*$^+$ sequencing primer
188 sim3-seq-3	GTCCTGGGAAATGCGTTGGG	*sim3*$^+$ sequencing primer
189 sim3-seq-5	GCAAAAGGCTGAGGAAAGCACG	*sim3*$^+$ sequencing primer
190 sim3-seq-2	TACCTTGAGTGACTAGTTGC	*sim3*$^+$ sequencing primer
191 sim3-seq-4	GAACGAGCCAATAGCTTCTGG	*sim3*$^+$ sequencing primer
192 sim3-seq-6	CAATAAATCGTAAATATCCGC	*sim3*$^+$ sequencing primer
193 sim3-seq-8	TGCATCTTTGACATAACAGC	*sim3*$^+$ sequencing primer
202 sim3-5-T7-kozak	GTTGTGTTTAATACGACTCACTAT AGGGCGAGAGCCACCATGTCTTC TGATACGAAAACACTG	primer to amplify *sim3*$^+$ with T7 kozak sequence for use in *in vitro* transcription/translation reaction
300 sim3-5-Xho1	TACTACCTCGAGATGTCTTCTGAT ACGAAAACA	primer to clone *sim3*$^+$ into pREP41X expression plasmid
301 sim3-3-BamH1	TACTACGGATCCTTAATCCTTCTT TTTCTTATC	primer to clone *sim3*$^+$ into pREP41X expression plasmid
26 TM1A	AACAATAAACACGAATGCCTC	primers to amplify central
27 TM1B	ATAGTACCATGCGATTGTCTG	core of cen1
33 FBPA	AATGACAATTCCCCACTAGCC	primers to amplify the *fbp1*
34 FBPB	ACTTCAGCTAGGATTCACCTGG	gene
31 OTRA	CACATCATCGTCGTACTACAT	primers to amplify the
32 OTRB	GATATCATCTATATTTAATGACTACT	outer repeats of cen1
IMRA	GGCTACCAGCATTGTTATTCATAA	primer to amplify the inner-
IMRB	GGATATATGTATTCTTGCACTC	most repeats of cen1
cenF	GAAAACACATCGTTGTCTTCAGAG	primers to amplify
cenR	CGTCTTGTAGCTGCATGTGAA	transcribed region of outer

		repeats of cen1
98 UpALKS_for	GTAAAACGACGGCCAGT	5' primer to sequence pAL-KS plasmid
99 UpALKS_rev	AACAGCTATGACCATGA	3' primer to sequence pAL-KS plasmid
PM001a-L	GTCGACGTTAGAACGCGGCTAC	primers to sequence DmNASP IMAGE clone LD44305
PM002a-R	GGGTTAAATTCCCGGGTACTGC	
pOT2-T7	AATACGACTCACTATAGG	primers to sequence DmNASP IMAGE clone LD44305
pOT2-PM001	CGTTAGAACGCGGCTACAAT	
NASP-3-EcoR1	TACTACGAATTCATGTCTGCTGA AGCCGAAGCAATC	primer to clone DmNASP into pGEX-4T-1
NASP-5-Xho1	TACTACCTCGAGCTAGACGGCG GCACGCTTGGC	primer to clone DmNASP into pGEX-4T-1
CID-5-BamH1	TACTACGGATCCATGCCACGAC ACAGCAGAGCC	primer to clone CENP-A^{CID} into pGEX-4T-1
CID-3-Xho1	TACTACCTCGAGCTAAAATTGCC GACCCCGGTC	primer to clone CENP-A^{CID} into pGEX-4T-1
DmH3-5-EcoR1	TACTACGAATTCATGGCTCGTAC CAAGCAAACTGCT	primer to clone DmH3.3 into pGEX-4T-1
DmH3-3-Xho1	TACTACCTCGAGTTAAGCACGCT CGCCGCGAATGCG	primer to clone DmH3.3 into pGEX-4T-1
DmH4-5-EcoR1	TACTACGAATTCATGACTGGTCG TGGTAAAGGAGGC	primer to clone DmH4 into pGEX-4T-1
DmH4-3-Xho1	TACTACCTCGAGTTAACCGCCAA ATCCGTAGAGGGTG	primer to clone DmH4 into pGEX-4T-1
DmH1-5-EcoR1	TACTACGAATTCATGTCTGATTCT GCAGTTGCAACG	primer to clone DmH1 into pGEX-4T-1
DmH1-3-Xho1	TACTACCTCGAGTTACTTTTTGGC AGCCGTAGTCTT	primer to clone DmH1 into
DmNASP-5-T7-kozak	GTTGTGTTTAATACGACTCACTAT AGGGCGAGAGCCACCATGTCTG CTGAAGCCGAAGC	primer to amplify DmNASP with T7 kozak sequence for use in *in vitro* transcription/translation reaction
DmNASP-3-Sal	TACTACGTCGACCTAGACGGCG GCACGCTTGGC	reverse primer to amplify DmNASP
CID-5-T7-kozak	GTTGTGTTTAATACGACTCACTAT AGGGCGAGAGCCACCATGCCAC GACACAGCAGAGCC	primer to amplify CENP-A^{CID} with T7 kozak sequence for use in *in vitro* transcription /translation reaction
CID-3-Xho1	TACTACCTCGAGCTAAAATTGCC GACCCCGGTC	reverse primer to amplify CENP-A^{CID}

CID_seq1	CGACTTTGAGTTGTTCGCCGAC	CENP-A^{CID} sequencing primer
CID_seq2	ACGACACGGCCTTCCGCTCGCC	CENP-A^{CID} sequencing primer
CID_seq3	GGAGAATCGCTATCCCACAACC	CENP-A^{CID} sequencing primer
CID_seq4	TTCGACGCGTCTGCGGCGATCT	CENP-A^{CID} sequencing primer
CID_seq5	CATGGCCAATAGGGCGCCTTCG	CENP-A^{CID} sequencing primer
CID_seq6	GTGCGAGATGTACTTGACGCAG	CENP-A^{CID} sequencing primer
T7-5-DmSim3	TTAATACGACTCACTATAGGGAG ATTGCAATGGCCTTGGACG	5' primer to amplify DmNASP DNA template for dsRNA synthesis
T7-3-DmSim3	TTAATACGACTCACTATAGGGAG AGCGTGCCGCCTCTAGTAT	3' primer to amplify DmNASP DNA template for dsRNA synthesis
EB-T7-5	GAATTAATACGACTCACTATAGG GAGAATGGCTGTAAACGTCTACT CCACAAATGTG	5' primer to amplify DmEB1 DNA template for dsRNA synthesis
EB-T7-3	GAATTAATACGACTCACTATAGG GAGATGCCCGTGCTGTTGGCAC AGGCGTTTA	3' primer to amplify DmEB1 DNA template for dsRNA synthesis
NASP_seq1	GTCATCATCATCGGCAGCCTCG	DmNASP sequencing primer
NASP_seq2	GATGACGAGCGACCGAGCACAT	DmNASP sequencing primer
NASP_seq3	CGCTTGATTGACGCAGTGCTG	DmNASP sequencing primer
NASP_seq4	GCAGGCCCAGACCATTGCTGAA	DmNASP sequencing primer
313 pINV-5-BamH1	AAAAACTGCGGATCCACTTTTGA TCCGTTC	primer to clone invertase promoter into 'split URA' plasmid
314 pINV-3-Pst1	TACTACCTGCAGGCAAATCTTCA AAGTTAG	primer to clone invertase promoter into 'split URA' plasmid
315 GFP-5-Pst1	TACTACCTGCAGAGTAAAGGAGA AGAACTT	primer to clone GFP-CENP-A^{cnp1} into 'split URA' plasmid
318 Cnp1-3-EcoR1	TACTACGAATTCTCAAGCACCAC GAATCCT	primer to clone GFP-CENP-A^{cnp1} into 'split URA' plasmid
41 uraforward	GAGGGGATGAAAAATCCCAT	primer to amplify *ura4*$^{+}$

42 urareverse	TTCGACAACAGGATTACGACC	across the DS/E region
K905	CATGATGCATGCCATGGCATACG ATATATTACGG	5' *arg3*$^+$ sequencing primer
K906	GTCAGAGCATGCCATGGATGATG CACACTCTACT	3' *arg3*$^+$ sequencing primer
WA03	CCCACTGGCTATATGTATGCATTT	5' primer upstream of *ura4*$^+$ UTR
PINV-seq1	CCGCCATTGCACTCAGCCATAC	reverse primer within invertase promoter
GFP_for GFP_rev	GTAAAGGAGAAGAACTTTTCACTG TCATCCATGCCATGTGTAATC	primers to amplify 800 bp GFP probe for Northern blot

CHAPTER 3

SCREEN TO ISOLATE MUTANTS THAT ALLEVIATE SILENCING AT BOTH THE CENTRAL DOMAIN AND OUTER REPEAT HETEROCHROMATIN: IDENTIFICATION AND INITIAL CHARACTERISATION OF *cos* MUTANTS

INTRODUCTION

Fission yeast centromeres are composed of two domains: the central core region (*cnt*), where the kinetochore assembles and the outer repeat region (*otr*), which is packaged as heterochromatin. Thus, fission yeast centromeres resemble those of vertebrates in that the kinetochore domain is found embedded in pericentric heterochromatin. Both domains are composed of silent chromatin and have molecularly and functionally distinct properties. The outer repeat sequences are packaged into regularly spaced nucleosomes, whereas the central domain chromatin lacks regular nucleosomal packaging and is nuclease resistant (Polizzi and Clarke, 1991, Takahashi et al., 1992). The outer repeat nucleosomes are underacetylated and methylated on lysine 9 of histone H3 and bound by the HP1 (heterochromatin protein 1) homologue Swi6 (Ekwall et al., 1997, Nakayama et al., 2001) and mediate sister chromatin cohesion through the formation of this pericentric heterochromatin and consequential recruitment of cohesin (Bernard et al., 2001, Nonaka et al., 2002). In contrast, the central domain is not packaged as heterochromatin and most nucleosomes of the central domain contain the centromere-specific histone H3-like variant CENP-A^{Cnp1} (Takahashi et al., 2000). Some histone H3 containing nucleosomes are also associated with the central core domain and these nucleosomes are dimethylated on lysine 4 of histone H3 (Cam et al., 2005), which is a mark of active transcription (Santos-Rosa et al., 2002, Bernstein et al., 2002, Schneider et al., 2004).

Both central core and outer repeat regions are required to form a fully functional centromere. It has been demonstrated by transformation of a mini-chromosome into cells that the establishment of an active centromere requires both central core sequences and at least one outer (K-type, K ≡ *dg* as described in chapter 1) repeat (Hahnenberger et al., 1991; Takahashi et al., 1992). Further studies have shown that each additional tandem copy of the K-type outer repeat increases the mitotic stability of a mini-chromosome (Baum et al., 1994). These additional repeats may stabilize the mini-chromosome perhaps by recruiting a higher density of cohesion, through increased binding of the Rad21 cohesin subunit to the centromere (Bernard et al., 2001, Nonaka et al., 2002) and allow sister kinetochores to face away from each other and to be segregated in a more efficient manner. Alternatively, the outer repeats may have an additional role in the specification of the site of kinetochore assembly and may provide the contextual specificity that defines a functional centromere.

Marker genes inserted into fission yeast centromeres are transcriptionally silenced (Allshire et al., 1994, 1995) and alleviation of silencing at either the central core or outer repeat heterochromatin has been correlated with loss of centromere function (Allshire et al., 1995, Pidoux et al., 2003, Ekwall et al., 1995, 1996). In addition it has been observed that mutants affecting silencing at the central core have no effect on silencing at the outer repeats. Moreover, most mutants affecting silencing at the outer repeats have little or no effect on silencing at the central core. For example, cells lacking Swi6 display alleviation of silencing at the outer repeats of the centromere with no effect on the central core (Allshire et al., 1995), whereas mutated alleles of the kinetochore component *mis6* alleviate silencing at the central core of the centromere but silencing at outer repeat heterochromatin is unperturbed (Partridge et al., 2000). There is also a correlation between the loss or mutation of a protein, which results in alleviation of silencing at a particular domain, and the ability of that protein to bind to the domain. Mutants such as *swi6Δ* lead to alleviation of outer repeat silencing and Swi6 protein was found to bind to the outer repeats only and not to the central core region (Allshire et al., 1995). In addition, mutants such as *mis6* and *sim4* specifically alleviate central core silencing (Partridge et al., 2000, Pidoux et al., 2003) and Mis6 and Sim4 proteins are specifically associated with the central core domain (Saitoh et al., 1997, Pidoux et al., 2003). Indeed, mutations in the centromeric histone H3 variant *CENP-A*^{cnp1} itself lead to alleviation of central core silencing (Pidoux et al., 2003).

It is now known that centromeric outer repeats are transcribed and that the RNA interference machinery is required for the establishment and maintenance of silent chromatin over the repetitive sequences (*dg-dh*) at the outer regions of the centromere (Volpe et al., 2002, reviewed in Grewal and Moazed, 2003). In accordance with this, mutations in components of the RNAi machinery in fission yeast, including the RNAse III-like enzyme Dicer 1 (Dcr1), the PIWI/family protein Argonaute 1 (Ago1) that targets mRNA for degradation and also the RNA-dependent RNA polymerase (Rdp1) have been shown to alleviate silencing specifically at the heterochromatic outer repeats (Volpe et al., 2003), but have little or no effect on central core silencing. This suggests that functionally distinct complexes assemble at or act on the two centromeric domains.

The transition between the domain where outer repeat proteins assemble, and the domain where central core proteins associate, is demarcated by tRNA genes present within the inner repeat sequences (*imr*) of all three centromeres (Takahashi et al., 1991). These tRNA genes have been shown to act as barriers, which separate the two domains and prevent the spreading of pericentric heterochromatin into the specialized central core chromatin (Scott et al., 2006). Intriguingly, lack of the tRNA chromatin barrier genes leads to abnormal chromosome segregation in meiosis (Scott et al., 2006) and so the presence of a barrier which separates the central domain from the flanking heterochromatin may be required for

proper centromere function. In addition, mini-chromosomes that lack these tRNA genes require the addition of spacer DNA for function, suggesting the spatial separation of the domains may also important (Baum et al., 1994). It is noted that human CENP-A is only assembled at a portion of the repetitive satellite DNA that comprise human centromeres (Warburton et al., 1997, Blower et al., 2002, Lam et al., 2006) and it is possible barrier elements may exist at centromeres of higher eukaryotes to restrict CENP-A assembly to certain regions.

It is clear that neither the central domain, nor the outer repeat domain alone, are sufficient for active centromere formation, but both are necessary. As a result, it is proposed here that it is the cross talk between the two domains that leads to functional centromere formation. Previously, mutations in seven loci (*sim1* to *7*; silencing in the middle of the centromere) have been identified, which specifically alleviate silencing at the central core domain but not at the outer repeats (Pidoux et al., 2003). One of these mutants, *sim6-86*, was unusual in that it affected silencing at the outer heterochromatic repeats and silencing at the central domain (Pidoux A, unpublished results). As this points to a possible link between the integrity of neighbouring heterochromatin, kinetochore assembly and the importance of maintaining silencing at both these domains, a second screen was implemented to identify *sim6-like* mutants.

In this chapter, I will describe a screen, which was implemented to identify mutants that alleviate both central core and outer repeat silencing. From this screen, the *cos* mutants (central core and outer repeat silencing) were identified. The newly isolated *cos* mutants may give insight into functional interplay between these two domains and perhaps a role for heterochromatin in orchestrating the deposition of CENP-A^{Cnp1} at centromeres. I will discuss the phenotype of the *cos* mutants isolated and focus on the characterisation of the *cos1* mutant and its role in chromosome segregation and centromere silencing.

RESULTS

3.1 A sensitive assay to monitor central core and outer repeat silencing

A strain was constructed previously that contained a promoter-crippled *arg3*$^+$ gene at the central core of centromere 1 (*cen1*) to monitor alleviation of central core silencing [*cnt1:arg3*$^+$], *ura4*$^+$ at the outer repeat of centromere 2 (*cen2*) to monitor alleviation of outer repeat silencing [*otr2:ura4*$^+$], a promoter-crippled *his3*$^+$ gene at telomere 1 (*tel1L*) to monitor alleviation of silencing at telomeres [*his3:tel1*$^+$] and *ade6*$^+$ at the central core of *cen3* [*cnt3:ade6*$^+$] (as described in Pidoux et al., 2003). Strains with the genotype *cnt1:arg3*$^+$ *otr2:ura4*$^+$ *cnt3:ade6*$^+$ *his3:tel1*$^+$ are referred to as wild type (3027 h$^+$/3033 h$^-$). Wild type strains containing *cnt1:arg3*$^+$ grow very slowly on media lacking arginine (-arg), forming

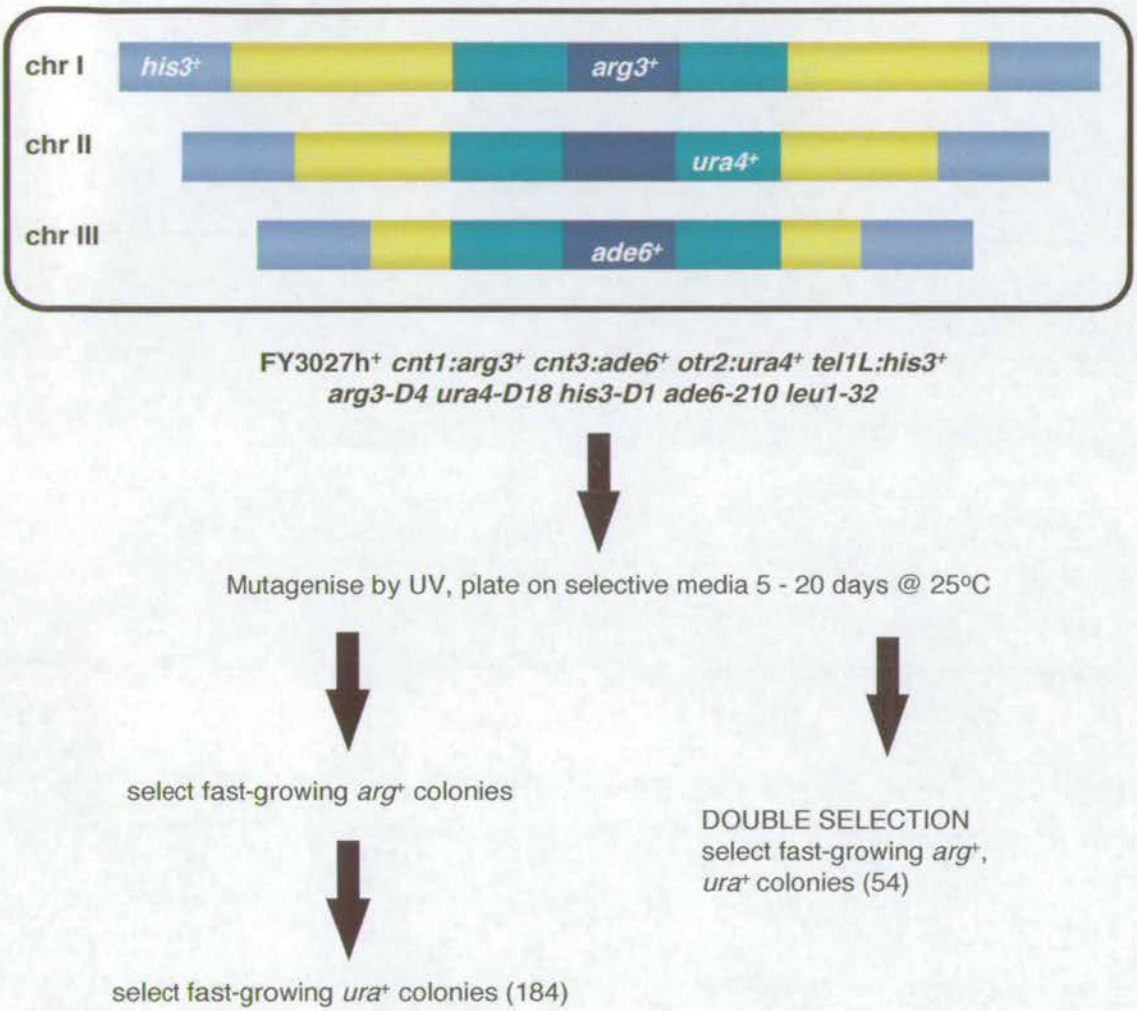


Figure 3-1. Schematic representation of wild type strain (3027h$^+$/3033h$^-$) used to isolate mutants defective in central core and outer repeat silencing.

The three fission yeast chromosomes and insertion of marker genes used to assay silencing are indicated. 3027/3033 contains a promoter-crippled *arg3*$^+$ gene at the central core of centromere 1 (dark blue) to monitor alleviation of central core silencing [*cnt1:arg3*$^+$], *ura4*$^+$ at the outer repeat of centromere 2 (green) to monitor alleviation of outer repeat silencing [*otr2:ura4*$^+$], a promoter-crippled *his3*$^+$ gene at telomere 1 (light blue) to monitor alleviation of silencing at telomeres [*his3:tel1*$^+$] and *ade6*$^+$ at the central core of *cen3* [*cnt3:ade6*$^+$] (as described in Pidoux et al., 2003). Scheme illustrates method used to select for *cos* mutants.

tiny colonies after several days' incubation at 25°C (Pidoux et al., 2003) (Figure 3-1).

3.2 Isolation of central core and outer repeat silencing (*cos*) mutants

The wild type strain (3027 h^+ / 3033 h^-) was plated either onto medium lacking arginine (-arg) and then onto medium lacking uracil (-ura) or directly on to double selection medium lacking both arginine and uracil (-arg, -ura) and mutagenised by UV (3-5 mJ, 50-80% killing). Plates were incubated at 25°C for 5 - 20 days and fast growing colonies (183 colonies from –arg plates, 54 colonies from -arg, -ura plates) were picked and streaked onto –arg plates to retest for alleviation of silencing. Fast growing colonies were subsequently picked from these –arg plates and streaked onto –ura plates to assay alleviation of silencing at outer repeats (*otr*). In total, 12 *sim6-like* mutants that alleviate silencing at both the central core and the outer repeat domains were isolated. *sim6-like* mutants were renamed *cos* mutants (central core and outer repeat silencing). 8 mutants that alleviate silencing at the central core only, and 4 mutants which alleviate silencing at the outer repeats only, were also isolated but were not further analysed (not shown) (Figure 3-1).

cos mutants were backcrossed three times to the wild type strain in order to confirm the mutation was in a single gene. After backcrossing, *cos* mutants were then retested for alleviation of silencing on selective media (-arg, -ura) and also for alleviation of telomeric silencing (-his). Mutants were not capable of growth on -his media (with the exception of *cos3-24*), indicating that silencing of the *his3*$^+$ gene inserted at the heterochromatic telomeres is maintained. Thus, the mutants did not display a general alleviation of silencing, but specifically alleviate silencing at the central core region and centromeric heterochromatin. Mutants were crossed to each other to test for allelism and placed into 5 linkage groups (*cos1 to 5*). *sim6-86* was renamed *cos1-86*. Four temperature sensitive (ts) alleles of *sim6/cos1* were isolated from the screen; *cos1-7*, *cos1-22*, *cos1-38*, *cos1-17*, along with four non ts alleles of *cos2*; *cos2-10*, *cos2-26*, *cos2-28*, *cos2-33*. As single mutant alleles (*cos3-24*, *cos4-35* and *cos5-13*) were isolated in three linkage groups, it suggests that the screen has not been saturated (Table 1).

3.3 Analysis of *cos1* mutants

cos1-86 and the newly isolated *cos1* alleles were subjected to further analysis.

3.3.1 Silencing defects and temperature sensitive phenotype

cos1 mutants alleviate both central core (could grow on medium lacking arginine) and outer repeat silencing (could grow on medium lacking uracil). *cos1* mutants could not grow on medium lacking histidine, and so do not affect silencing at telomeres, which are also

Comp Group	Mutant	Growth on -ARG	Growth on -URA	Growth on -HIS	Temperature sensitive	TBZ sensitive
1	*cos1-86*	+	+	-	+	+
	cos1-7	+	+	-	+	+
	cos1-22	+	+	-	+	+
	cos1-38	+	+	-	+	+
	cos1-17	+	+	-	+	+
2	*cos2-10*	+	+	-	-	+
	cos2-26	+	+	-	-	+
	cos2-28	+	+	-	-	+
	cos2-33	+	+	-	-	+
3	*cos3-24*	+	-	+	-	-
4	*cos4-35*	+	+	-	-	+
5	*cos5-13*	+	+	-	-	+

Table 1. Summary of *cos* mutant phenotypes.

Strains were backcrossed three times to wild type strain and placed into 5 linkage groups. Strains were assayed for growth on medium lacking arginine (-ARG) to test for alleviation of central core silencing, on medium lacking uracil (-URA) to test for alleviation of outer repeat silencing and growth on medium lacking histidine (-HIS) to test for alleviation of silencing at telomeres. Temperature sensitivity was assayed by growth on Phloxin B at 36°C. Sensitivity to microtubule destabilisation was assayed using TBZ (10 μg/ml).

packaged as silent heterochromatin in fission yeast and so specifically alleviate centromere silencing (Figure 3-2A). In addition, *cos1* mutants, which had a *ura4*$^+$ marker gene inserted at the outer repeats, were found to be sensitive to growth on 5-fluoro-orotic acid (5-FOA, a counter-selective drug which selects for cells which are expressing *ura4*$^+$). *cos1* mutants are temperature sensitive (ts) and fail to form colonies on phloxin at 36°C. Phloxin is a dye, which can be taken up and exported by healthy cells. Dead cells become a dark pink in colour as they are able to take up the dye but are unable to export it. Thus, phloxin dye may be used as an assay to indicate temperature sensitivity.

3.3.2 Outer repeat silencing defects

In fission yeast, the *ade6*$^+$ gene provides a convenient silencing assay as changes in its expression can be monitored directly by changes in colony colour (Allshire et al., 1994). Genes inserted within heterochromatic regions, such as the outer repeats of the centromere, mating type loci and telomeres are transcriptionally repressed. In wild type fission yeast cells, repression of the *ade6*$^+$ gene renders cells *ade6*$^-$, resulting in the accumulation of a substrate of adenine metabolism that, when exposed to oxygen, confers a red colour to cells grown on low adenine supplemented plates (Fisher, 1969). In mutants such as *clr4*Δ or *rik1*Δ, where heterochromatin is disrupted, silencing at outer repeats is derepressed, allowing expression of the *ade6*$^+$ gene and formation of white colonies on the limited adenine plates (Ekwall et al., 1997, Ekwall et al., 1999).

To further investigate silencing defects at outer repeats, *cos1* mutants were crossed to a strain with the *ade6*$^+$ marker gene inserted at the outer repeats of centromere 1 (FY1180 *cen1otr1R*(Sph1):*ade6*$^+$, Figure 3-3) and the endogenous *ade6*$^+$ gene is mutated (*ade6-210*) (Ekwall et al., 1997). As shown in Figure 3-4, *rik1*Δ cells strongly alleviate repression, allowing the expression of the *ade6*$^+$ and the formation of white colonies in the low adenine plates. All *cos1* mutant alleles were pale pink in colour, however none of the *cos1* ts mutants were found to display silencing defects as strong as *rik1*Δ, which is white in colour. This is consistent with the finding that *cos1* mutants also alleviate silencing of the *ura4*$^+$ gene inserted at the outer repeat of centromere 2 (*cen2*) and were sensitive to FOA, as described in Figure 3-2A. The result of the colony colour assay confirms that transcriptional state of the normally silent *ade6*$^+$ marker gene inserted at the outer repeat domain is disrupted in *cos1* mutants. However, the alleviation of silencing at heterochromatin is intermediate and not to the same degree as that exhibited by mutants of the heterochromatin component Rik1.

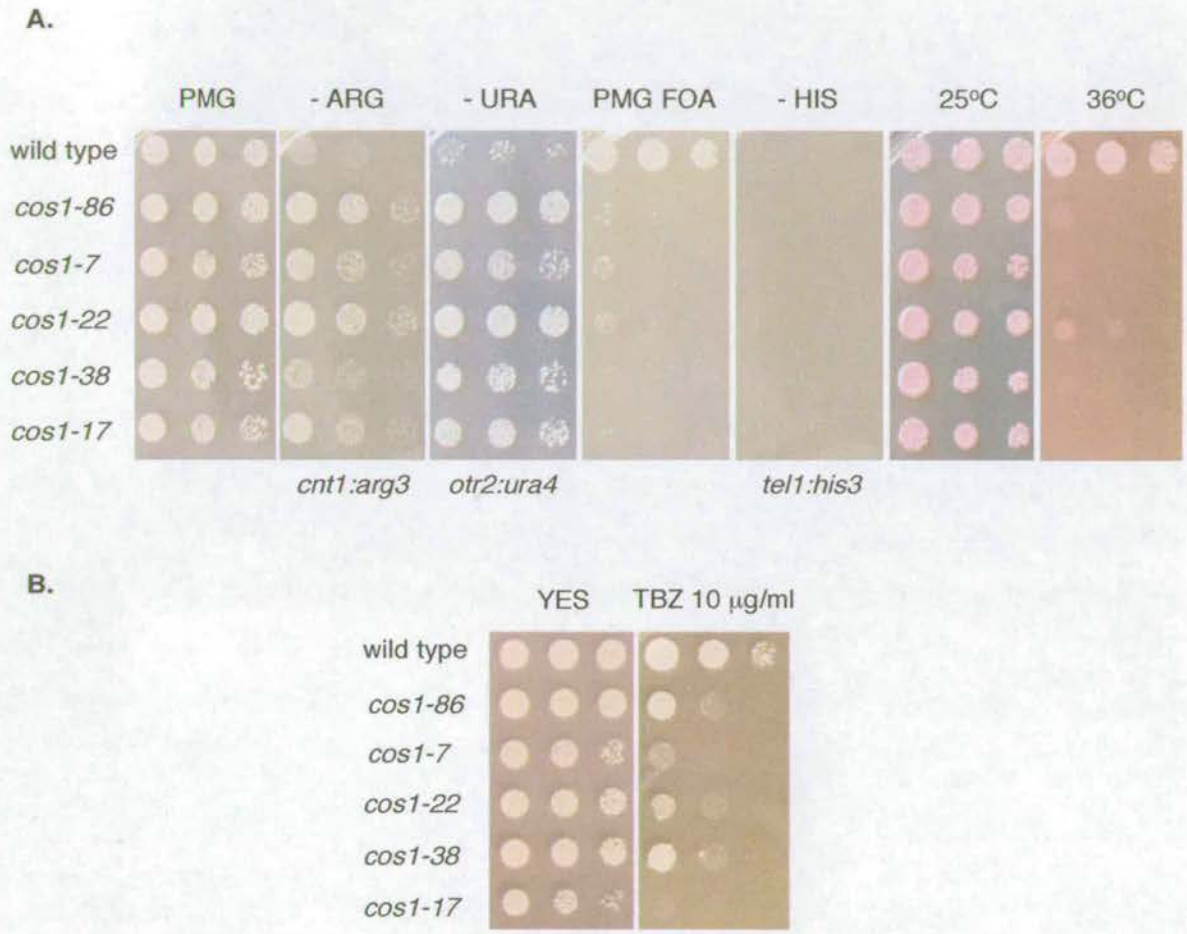


Figure 3-2. *cos1* mutants alleviate central core and outer repeat silencing.

A. The wild type strain 3027 has an *arg3*$^+$ marker gene inserted at central core of centromere 1, a *ura4*$^+$ marker gene inserted at outer repeats of centromere 2 and a *his3*$^+$ marker gene inserted at telomere1. Wild type cells fail to grow on medium lacking arginine, lacking uracil or lacking histidine as silencing of the inserted marker genes is maintained. *cos1* mutant alleles alleviate silencing at the central core (grow on medium lacking arginine) and outer repeats (grow on medium lacking uracil) but do not affect silencing at the telomere (no growth on medium lacking histidine). *cos1* mutants were also sensitive to growth in PMG media containing FOA, a counter-selective drug for expression of *ura4*$^+$. *cos1* mutants are temperature sensitive and fail to form colonies on Phloxin B at 36°C.

B. *cos1* mutants are sensitive to the microtubule destabilising drug TBZ. Tenfold dilutions of cells were spotted onto YES complete media supplemented with 10 μg/ml TBZ.

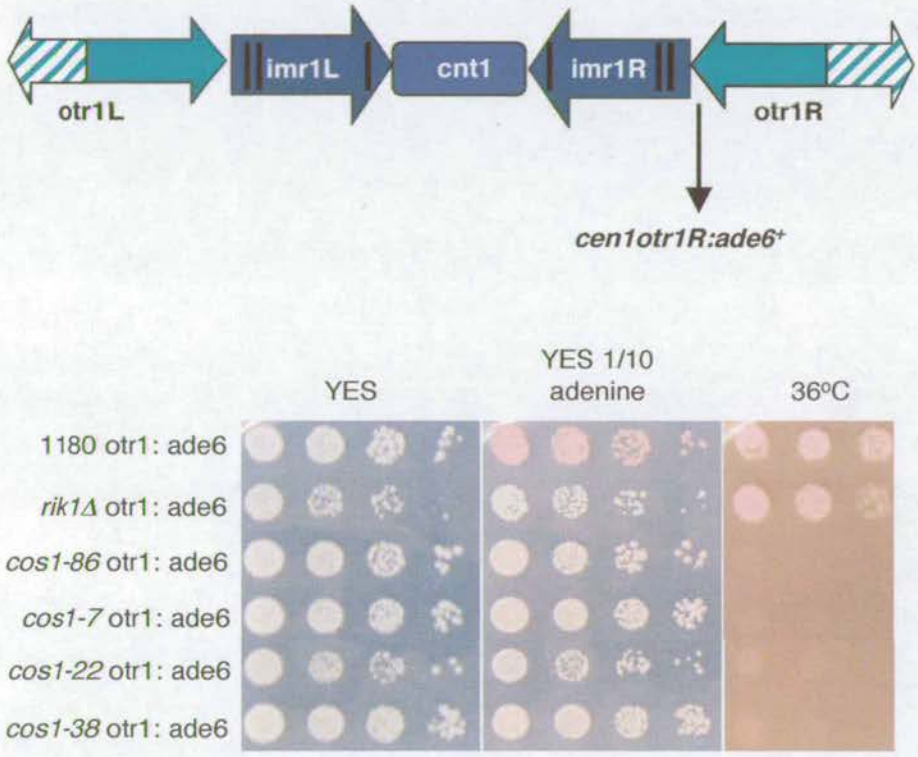


Figure 3-3. *cos1* mutants alleviate silencing of *ade6+* marker gene inserted at the outer repeats.

Colony colour assay was used to analyse the degree of defect in silencing at outer repeats. Strains were crossed to strain FY1180 that has an *ade6+* gene inserted at the outer repeats of centromere 1 (*cen1otrR(SphI):ade6+*) and were plated on low adenine supplemented YES plates. In wild type cells, *ade6+* is silenced and colonies are red in colour; in mutant strains that alleviate silencing at outer repeats, such as *rik1Δ*, the *ade6+* gene is expressed and colonies are white in colour. Using this assay, *cos1-86*, *cos1-7*, *cos1-22* and *cos1-38* were found to partially alleviate silencing at the outer repeats and were pink in colour. Temperature sensitivity of *cos1* mutants is shown by failure to form colonies on medium containing Phloxin B at 36°C.

3.3.3 *cos1* mutants display chromosome segregation defects including lagging chromosomes

cos1 mutants were found to show supersensitivity to the microtubule-disrupting drug TBZ (thiobendazole) (Figure 3-2B). Mutants in centromere function are often impaired in their ability to interact with microtubules and the presence of TBZ exacerbates this defect reducing cell viability but has only a very mild effect on wild type growth.

Mutant strains that alleviate central core silencing such as *sim4* or outer repeat silencing, such as *swi6*Δ, display defective chromosome segregation, with high rates of lagging chromosomes on late anaphase spindles (Allshire et al., 1995, Ekwall et al., 1995, Ekwall et al., 1996, Pidoux et al., 2000, Pidoux et al., 2003). To investigate whether *cos1* mutations result in chromosome segregation defects or abnormal cell morphology, *cos1* mutants and the isogenic wild type control strain (3027/3033) were harvested from log phase cultures grown at permissive and restrictive temperatures (6 hours at 36°C). Cells were fixed and stained with anti-α-tubulin to decorate microtubules and 4-, 6-diamidino-2-phenylindole (DAPI) to stain for DNA. Cells were then viewed and analysed for the presence of segregation defects (n=200). Segregation defects were rarely seen in the wild-type strain (approximately 0.5% at 25°C and 36°C). *cos1-86* and newly isolated alleles (*cos1-7*, *cos1-22*, *cos1-38*, *cos1-17*) displayed multiple forms of chromosome mis-segregation, including lagging chromosomes at anaphase and unequal segregation of DNA (Figure 3-4). The frequency of lagging chromosomes was counted for each *cos1* mutant allele, with *cos1-7* showing the highest degree of segregation defects (13.4% at 25°C and 52% at 36°C), (Table 2). Lagging chromosomes and unequal segregation may suggest a role for the *cos1*$^+$ gene product in establishing correct microtubule-kinetochore attachments or biorientation of the centromere.

A striking feature of *cos1-86*, *cos1-7*, *cos1-38* and *cos1-22* mutants (*cos1-17* was not assessed) was an elongated cell phenotype even at 25°C, which was greatly enhanced at the restrictive temperature (Figure 3-4). This long cell phenotype is indicative of a cell-cycle delay, presumably in G1, S or G2 phase as cells continue to grow are blocked/delayed in the cell cycle. A similar phenotype is also observed in DNA damage checkpoint mutants due to an arrest in cell cycle progression (reviewed by Caspari and Carr, 1999).

3.3.4 Cloning of *cos1*$^+$ and identification of *cos1* mutations

In order to clone the *cos1*$^+$ gene, a *LEU2*$^+$ *S. pombe* genomic library (gift from the Shimoda laboratory) was transformed into *cos1* mutants by electroporation and cells were screened for rescue of their ts phenotype (complementation of TBZ^S was also attempted but no

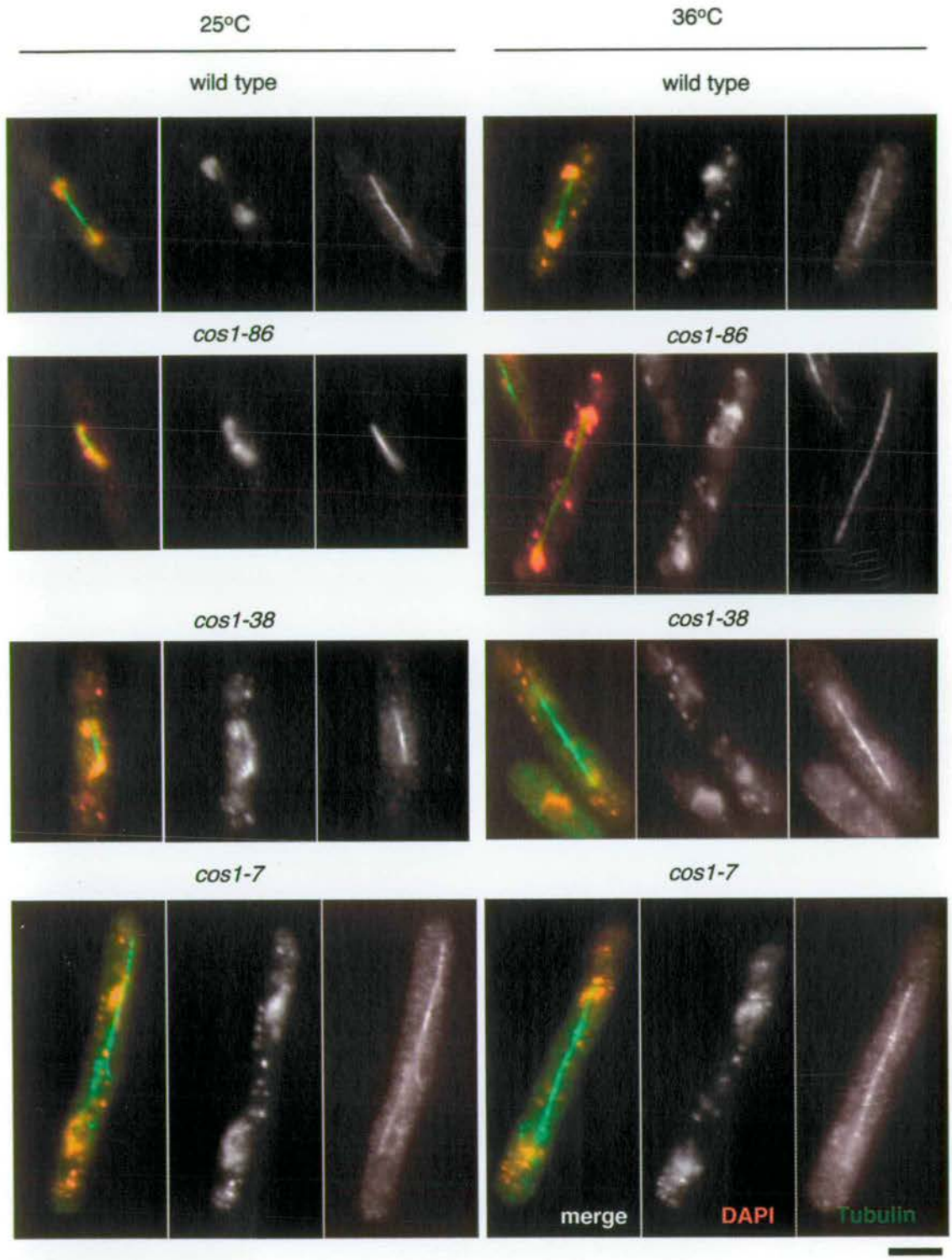


Figure 3-4. Chromosome segregation defects in *cos1* mutants.

Wild type and *cos1* mutant cells were grown at 25°C or at 36°C for 6 hours before fixing and staining with anti-α-tubulin (green) and DAPI stained DNA (red). Bar, 3 μm.

Strain	% Lagging chromosomes	
	Temperature	
	25°C	36°C
wild type	0.5	0.58
cos1-86	9.5	37
cos1-7	13.4	52
cos1-38	5	26
cos1-22	7.8	22.9

Table 2. Frequency of lagging chromosomes in *cos1* mutants.

Wild type and *cos1* mutant cells were grown at 25°C or at 36°C for 6 hours before fixing and staining with anti-α-tubulin and DAPI stained DNA. Lagging chromosomes were counted in cells with late anaphase spindles and are given as a percentage of total number of cells in anaphase (n=200). Lagging chromosomes were very rare in the wild type (0.5%-0.58%) at both temperatures.

positive colonies were obtained). This library consists of about 60,000 independent clones made as *Sau3A* cut genomic DNA fragments of an average size of 8 kb cloned into the *LEU2*$^+$ pAL-KS vector at the *BamHI site*. Transformants were plated on minimal medium lacking leucine and containing phloxin (0.02% v/v). After five days growth at 25°C, plates were shifted to 36°C for 1 - 2 days, to select for colonies that were now capable of growing at this temperature (Figure 3-5). At 36°C, 1 single colony out of 18,000 *cos1-86* colonies analysed was pale pink in colour and could grow at the restrictive temperature. *cos1-86* mutants that were transformed with empty pAL-KS plasmid remained temperature sensitive at 36°C.

The plasmid pb1E7 was rescued from the yeast, amplified in *E. coli* and recovered plasmid was transformed back into *cos1* mutants. Plasmid pb1E7 was found to complement the temperature sensitivity and silencing phenotypes of *cos-1-86* mutant and other *cos1* alleles (Figure 3-6). Partial sequencing of the plasmid using universal 5′ and 3′ primers annealing to the multiple cloning site of the pAL-KS plasmid, revealed two open reading frames (ORF's); one encoding a putative DNA-directed RNA polymerase III complex subunit (SPAPB1E7.03) and a 2,548bp ORF (SPAPB1E7.02) encoding *mcl1*$^+$.

3.3.5 *cos1* mutants have mutations in the *mcl1*$^+$ (mini chromosome loss 1) ORF

As *mcl1*$^+$ has been shown to be important for chromosome replication, cohesion and segregation (Williams et al., 2002) and displays phenotypes similar to *cos1-86* and newly isolated *cos1* alleles, it seemed likely that these mutants represent mutations in the *mcl1*$^+$ gene. To confirm this genetically, the plasmid containing *LEU2*$^+$ and *mcl1*$^+$ was linearised and integrated into the genome of the strain *cos1-86* at the endogenous *cos1*$^+$ locus and also in a wild type strain. The *leu*$^+$ -integrated strain (*cos1-86*^{INT} or wild type^{INT}) was then crossed to *cos1-86* and to isogenic wild type. The cross between *cos1-86*^{INT} (*leu*$^+$ and non ts) and *cos1-86* strain (*leu*$^-$ and ts) was examined by random spore analysis and 100% of *leu*$^+$ progeny were non temperature sensitive, demonstrating that the *leu*$^+$ marker and ts phenotype are tightly linked. Of the *leu*$^+$ colonies resulting from crossing wild type^{INT} to *cos1-86*, 50% were wild type and 50% were ts. No temperature sensitive progeny resulted from the cross of *cos1-86*^{INT} strain to wild type. This indicates that it is very likely that the SPAPB1E7.02 gene is the *cos1*$^+$ gene rather than a multi-copy suppressor (Figure 3-7).

In addition, genomic DNA was prepared from *cos1* mutant alleles and primers were designed every 200 bps in order to facilitate sequencing of the *mcl1*$^+$ ORF in wild type and *cos1* mutants. *cos1* mutants were found to have the following mutations in the *mcl1*$^+$ ORF: *cos1-7* has a G to A point mutation resulting in change of G (glycine) to R (arginine) at amino acid 242, *cos1-38* has a G to A mutation resulting in change of G (glycine) to E (glutamic acid) at amino acid 622 and *cos1-86* has a C to T mutation resulting in change

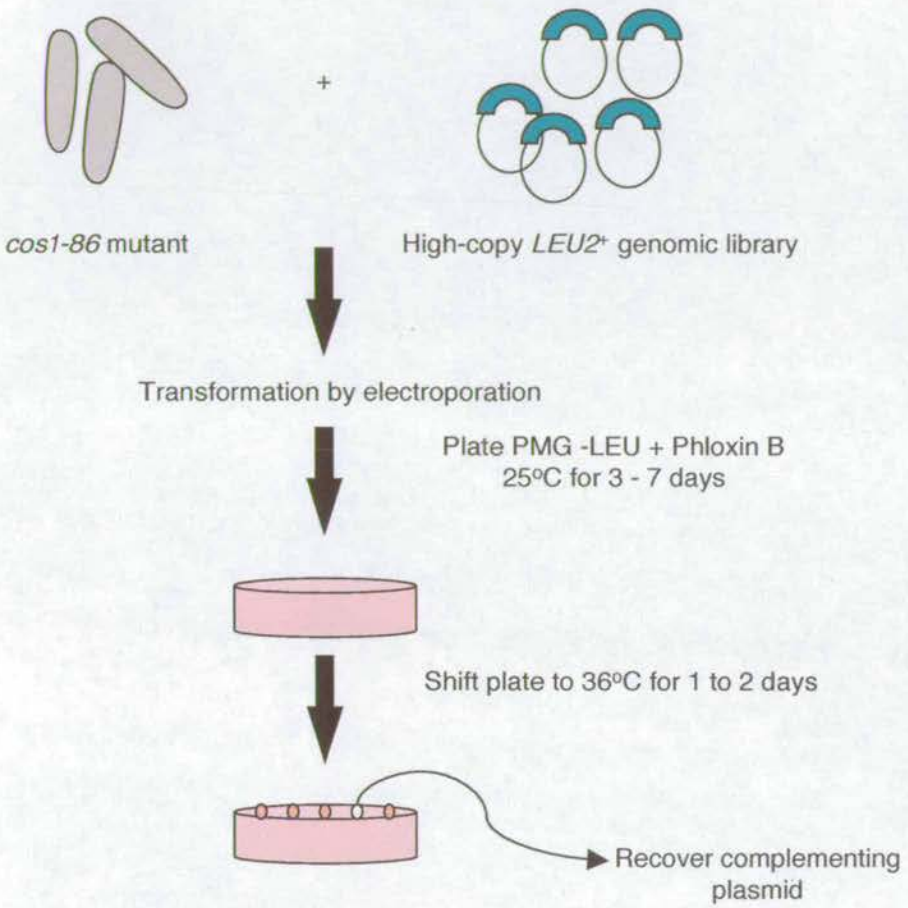


Figure 3-5. Strategy to clone *cos1*$^+$ by complementation of temperature sensitive phenotype using a high copy genomic library.

Mutants that remained temperature sensitive after 2 days at 36°C were dark pink in colour; complementation of ts by the library transformation resulted in the growth of cells that were white in colour (see text for details).

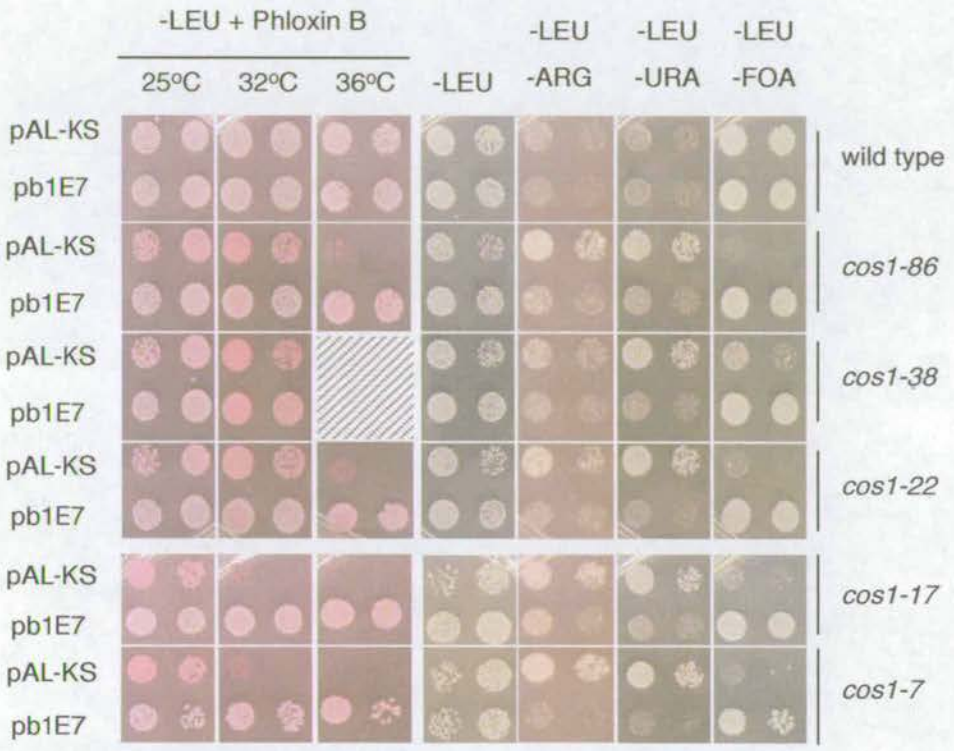


Figure 3-6. *cos1* mutants are complemented by pb1E7 plasmid encoding *mcl1*$^+$ ORF.

Plasmid pb1E7, that rescued the temperature sensitivity of *cos1-86*, was transformed into other *cos1* mutant alleles along with control empty *LEU2*$^+$ plasmid pAL-KS. In wild type cells (3027), the *arg3*$^+$ gene inserted at the central core and *ura4*$^+$ gene inserted at outer repeats are silenced and form tiny colonies on plates lacking arginine and uracil respectively. *cos1* mutants alleviate silencing at the central core and outer repeats and grow on medium lacking arginine and uracil. Transformation of *LEU2*$^+$ pb1E7 into *cos1* mutants and spotting onto medium lacking arginine (-LEU-ARG) and lacking uracil (-LEU-URA) resulted in complementation of silencing phenotypes. FOA is a counter selective media that selects for *ura4*$^-$ cells. Temperature sensitivity of *cos1* mutants was also complemented as assayed by growth of light pink colonies on Phloxin B at 36°C.

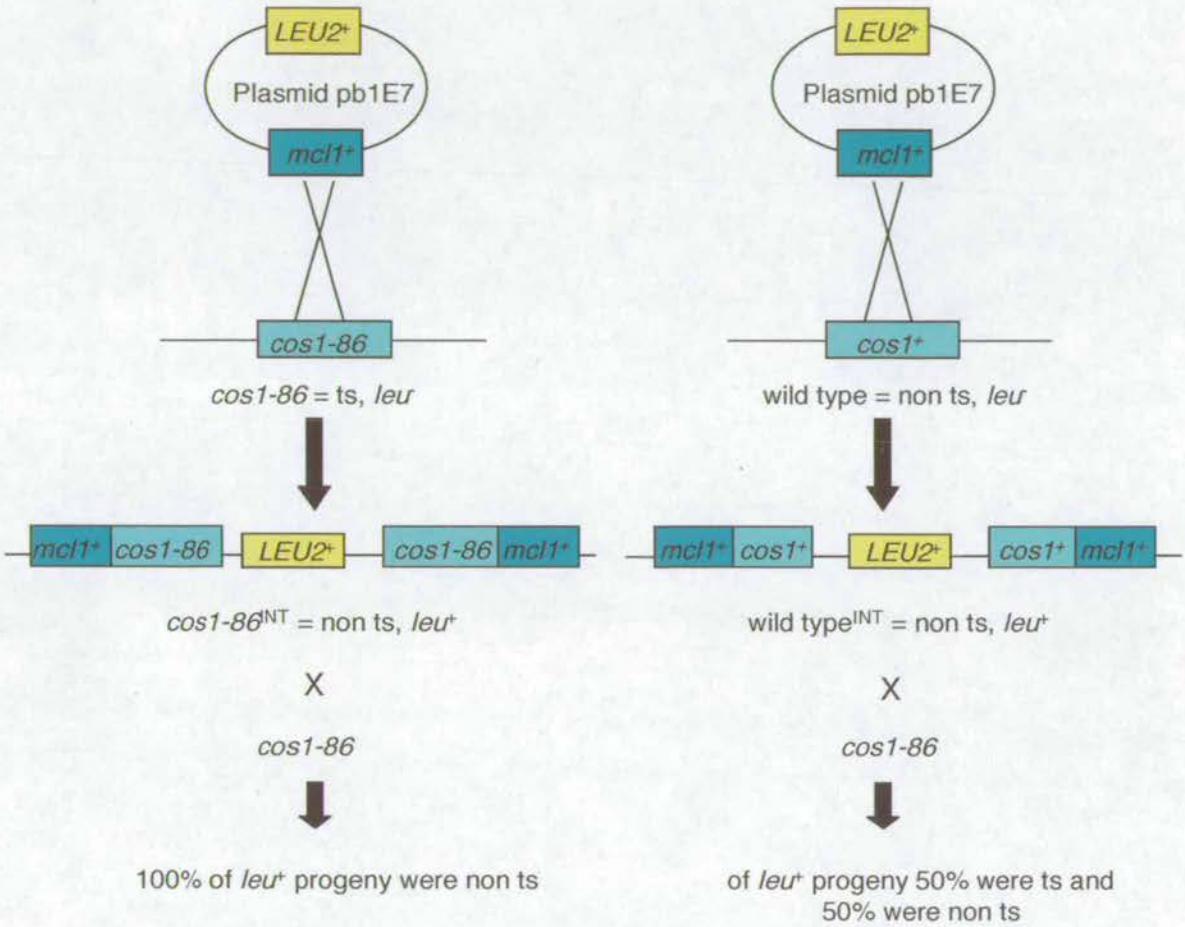


Figure 3-7. *cos1-86* is complemented by *mcl1+* (pb1E7).

pb1E7 was linearised by restriction digest within the *mcl1+* gene and transformed into *cos1-86* mutant (*cos1-86*^{INT}) and into wild type (*wild type*^{INT}) for integration at *mcl1+* genomic locus. Integrated strains were then crossed to *cos1-86* mutant and tight linkage between the *leu+* marker and non ts phenotype was demonstrated.

from a Q (glutamine) to a STOP codon at amino acid 765 (Figure 3-8A and 3-8B).

mcl1$^+$ encodes a 815-aa, highly acidic, 90.9 kDa protein composed of 5 WD40 repeats (WD for tryptophan and aspartic acid, also know as β-transducin domains) in the N terminus, which are involved in protein-protein interactions and three highly conserved regions, designated sepB boxes (Williams et al., 2002), of unknown function in the C terminus (see Figures 3-8 and 3-9). *mcl1*$^+$ is the *S. pombe* homologue of *S. cerevisiae CTF4* (Williams et al., 2002). *CTF4* was identified in budding yeast by two distinct approaches: a genetic screen for mutants affecting chromosome transmission fidelity (*ctf4/chl15*) (Kouprina et al., 1992) and affinity chromatography with Polα to identify DNA polymerase 1 binding proteins (*POB1*) (Miles and Formosa, 1992). Mcl1 is also homologous to *Aspergillus nidulans* SepB, which is known to be involved in regulation of chromosome segregation and cytokinesis (Harris and Hamer, 1995). Similar proteins have been identified in higher eukaryotes, including the human DNA binding protein AND-1 which has 5 conserved WD40 repeats, 3 sepB boxes and in addition a HMG (high mobility group) domain (Kohler et al., 1997), (Figure 3-9).

A temperature sensitive *mcl1* allele, *mcl1-1* was found to show mitotic phenotypes including: septation prior to chromatin segregation ('cut'), an unequal assortment of DNA to daughter cells, lagging chromosomes, asymmetric spindle placement in the cell and chromatin bridges (Williams et al., 2002). Williams and McIntosh (2002) reported that *mcl1*$^+$ is an essential gene, where *mcl1*Δ cells are extremely slow growing forming micro-colonies of long, cdc-like cells after a week of growth. Such long cells are reminiscent of the *cos1-86* and *cos1-7* ts alleles that were isolated from the *cos* screen. A separate study by Tsutsui et al. (2005) reported that *mcl1*Δ cells are temperature sensitive for growth at 36°C but are viable at 25°C, suggesting that *mcl1*$^+$ is not an essential gene. This discrepancy in the literature may be reconciled by the observation that *mcl1*Δ cells described by Tsutsui et al. (2005) show extremely poor viability even at 25°C. For this reason, studies performed on *mcl1* temperature sensitive point mutations may provide a more accurate report of *mcl1* phenotypes. Mcl1-GFP is a constitutive nuclear protein that associates with chromatin from G1 to S phase and is absent from cells that contain a mitotic spindle (Williams et al., 2002). Mcl1 has recently been shown to interact with Pof3, a component of the ubiquitin ligase complex SCF (Skp1/Cullin/F-box) however the role of Mcl1 in complex with Pof3 is unclear (Mamnun et al., 2006).

3.3.6 Levels of methylation of histone H3 on lysine 9 (H3K9me2) are maintained in *cos1* mutants

It is clear from previous analyses, and from this current study, that Cos1/Mcl1 plays an important role in chromosome segregation. Recently, Mamnun et al. (2006) reported that

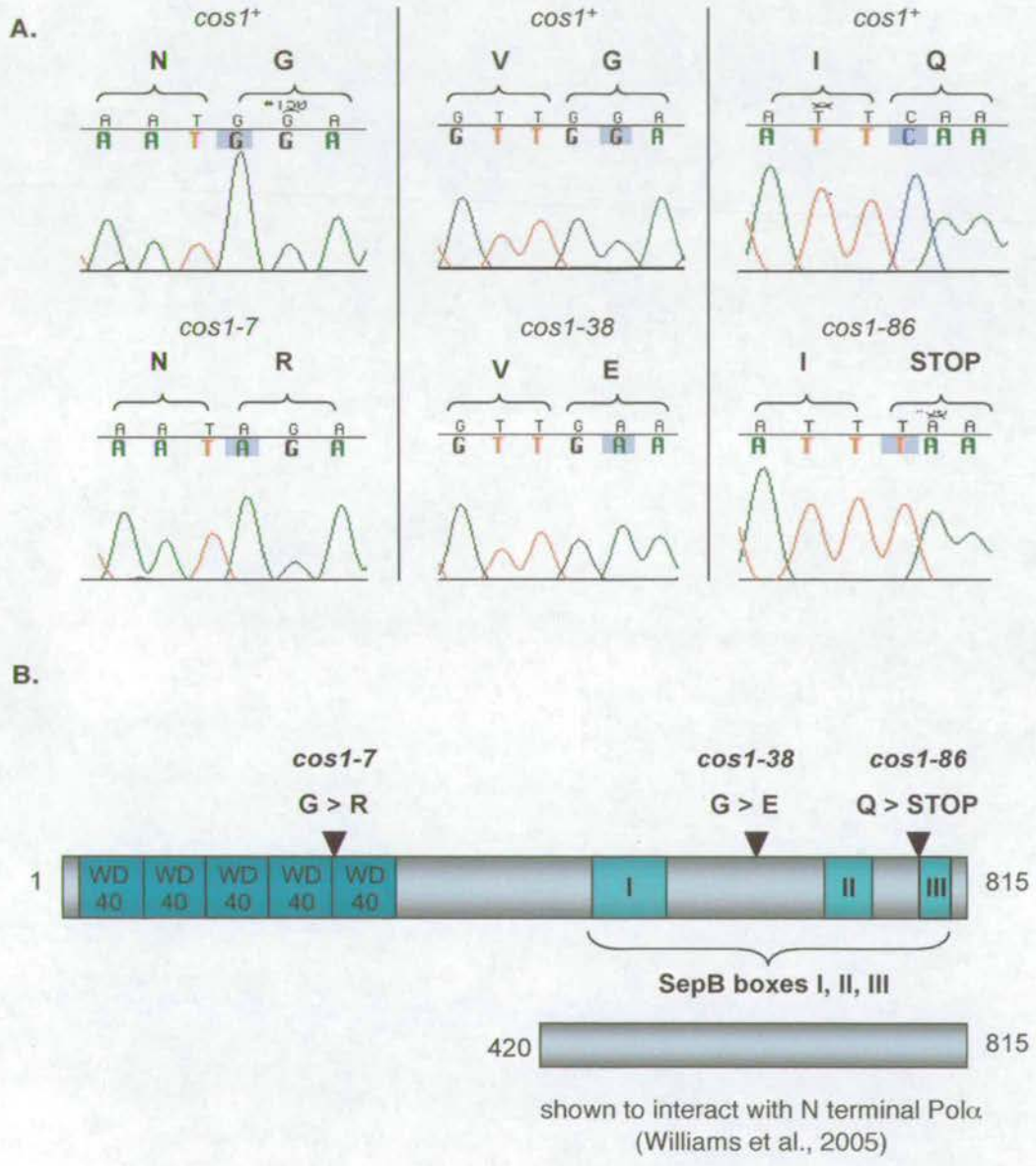
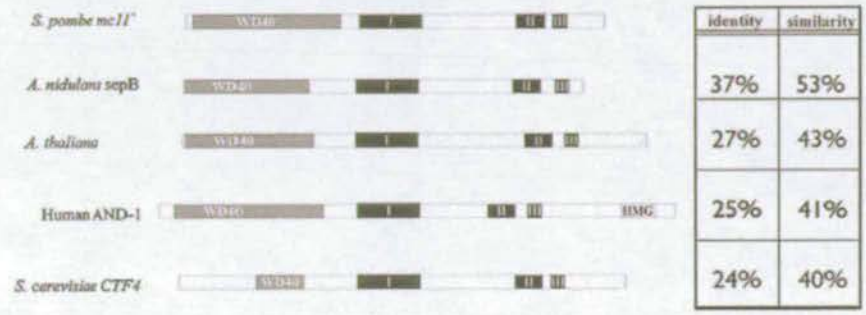


Figure 3-8. *cos1*⁺ is allelic to *mcl1*⁺.

A. Sequencing analysis was performed on *mcl1*⁺ gene from wild type and *cos1* mutants in both orientations. *cos1-7*, *cos1-38* and *cos1-86* point mutations are illustrated in chromatograms taken from sequence analysis using Sequencer 4.1 program.

B. Schematic showing domains of Mcl1 protein and location of *cos1* point mutations. Mcl1 has 5 WD40 (also called β-transducin) repeats at N terminus, thought to play a role in protein protein interactions, along with three conserved sepB boxes I, II and III of unknown function in the C terminus. The N terminal domain of Mcl1 which was shown to interact with Polα, is also indicated (Williams et al., 2005).

A.



identity	similarity
37%	53%
27%	43%
25%	41%
24%	40%

B.

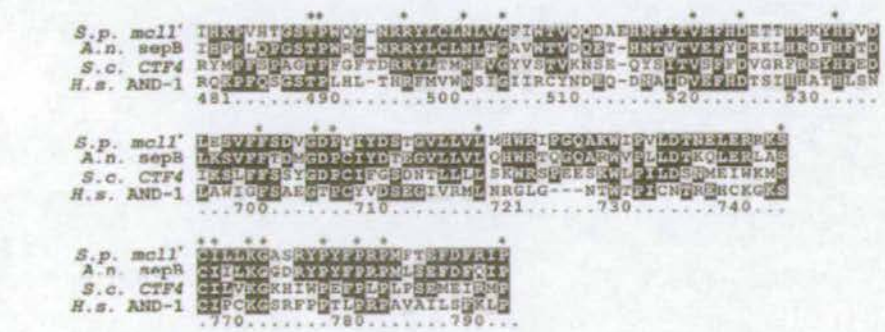


Figure 3-9. Mcl1 is a conserved eukaryotic protein (adapted from Williams et al., 2002).

A. BLAST analysis detected proteins with high similarity to *S. pombe* Mcl1 including *Aspergillius nidulans* (*A.nidulans* sepB), *Arabidopsis thaliana*, *Saccharomyces cerevisiae* (*S. c. CTF4*) and human (*H.s.* AND-1). The domain structure of Mcl1 and sequence comparison results among sepB family members are shown. Identity and similarity percentages are for comparisons over the entire length of the protein. HMG, high mobility group.

B. Clustal X alignment of putative family members revealed three highly conserved domains, designated SepB boxes, and are unique to this family of proteins.

*mcl1*Δ cells alleviate silencing of *ade6*$^{+}$ at the outer repeats and are white in colour. To understand further its possible role in silent centromeric heterochromatin formation, the level of methylation of histone H3 tails on lysine 9 in *cos1* mutants was investigated. Heterochromatin is formed at the outer repeats through the action of the Clr4 histone methyl transferase, which methylates histone H3 on lysine 9 (Rea et al., 2000; Nakayama et al., 2001) allowing the binding of Swi6 (Partridge et al., 2000; Bannister et al., 2001; Partridge et al., 2002). Swi6 is required to recruit cohesin and to mediate sister-chromatid cohesion (Bernard et al., 2001, Nonaka et al., 2002). These events lead to the formation of silent chromatin and deletion of *clr4*Δ and *swi6*Δ leads to alleviation of silencing at heterochromatin regions (Allshire et al., 1995, Ekwall et al., 1996, Bernard et al., 2000). Thus, loss of silencing at heterochromatic outer repeats has been correlated with a loss in H3K9me2 (dimethylation). As *cos1* mutants alleviate silencing at heterochromatin, chromatin immunoprecipitation experiments were performed with anti-H3K9me2 antibody to check if this marker for heterochromatin is intact. The immunoprecipitated DNA was analysed by multiplex PCR using primers, which amplify the centromeric outer repeat sequence (*cenF/R*) and primers that amplify the *fbp1* euchromatic gene (*fbp1*), which serves as a negative control. At both 25°C and after 6 hours at 36°C, *cos1-86* and *cos1-7* maintain wild type levels of H3K9me2 at outer repeats, whereas this enrichment was lost in the *clr4*Δ mutant negative control (Figure 3-10).

3.3.7 The histone H3 variant CENP-A^{Cnp1} is maintained at centromeres in *cos1* mutants, however some centromere declustering occurs in interphase

As *cos1* mutants also alleviate silencing at the central core domain, where the H3 variant CENP-A^{Cnp1} is specifically assembled, the localisation of CENP-A^{Cnp1} in *cos1* mutants was determined. Affinity purified anti-CENP-A^{Cnp1} antibodies were used to localise CENP-A^{Cnp1} in wild type and mutant *cos1* temperature sensitive alleles at 25°C and 36°C (Figure 3-11). Cells were co-stained with anti-Sad1, a spindle pole body marker (Hagan and Yanagida, 1995), which allowed the localisation of the clustered centromeres at the nuclear periphery to be determined (Funabiki et al., 1993). At both permissive and restrictive temperatures CENP-A^{Cnp1} localisation to centromeres appeared as wild type in *cos1* mutants. A recent study by Mamnun et al. (2006) reported that in *mcl1*Δ cells the dot-like pattern of CENP-A^{Cnp1}-GFP is lost and is distributed throughout the nucleus, indicating that the temperature sensitive alleles of *cos1* may not be fully penetrant at the restrictive temperature. As discussed above, *mcl1*Δ cells show extremely poor growth even at 25°C suggesting that these effects on CENP-A^{Cnp1} localisation could be indirect. Also, use of the tagged CENP-A^{Cnp1}-GFP may be more sensitive to changes in CENP-A^{Cnp1} localisation.

Anti-CENP-A^{Cnp1} staining in *cos1-86* and *cos1-7* mutants did however reveal that centromeres appeared to be slightly declustered at interphase at the restrictive temperature

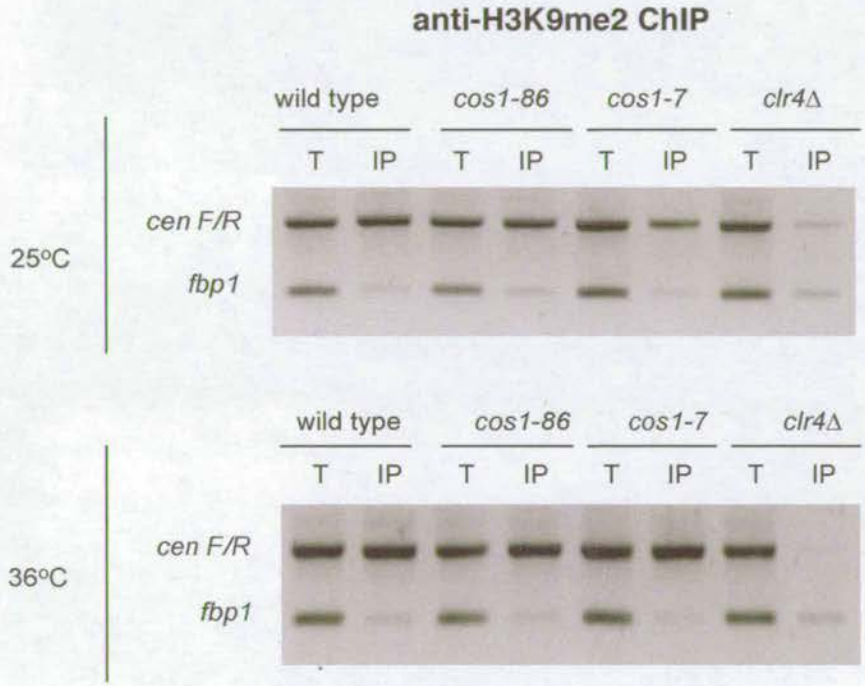


Figure 3-10. *cos1-86* and *cos1-7* maintain H3K9me2 at centromeric outer repeats.

Chromatin immunoprecipitation was performed on *cos1* mutants at permissive (25°C) and restrictive temperature (6 hours at 36°C) with antibodies specific for H3K9me2. The immunoprecipitated DNA was analysed in a multiplex PCR by primer pairs for the outer repeats (*cenF* and *cenR*) and a euchromatic control gene *fbp1*. *cos1-86* and *cos1-7* retained levels of H3K9me2 similar to wild type at permissive and restrictive temperatures. H3K9me2 is completely abolished in *clr4Δ* cells, used as a negative control. T = Total, IP = Immunoprecipitate.

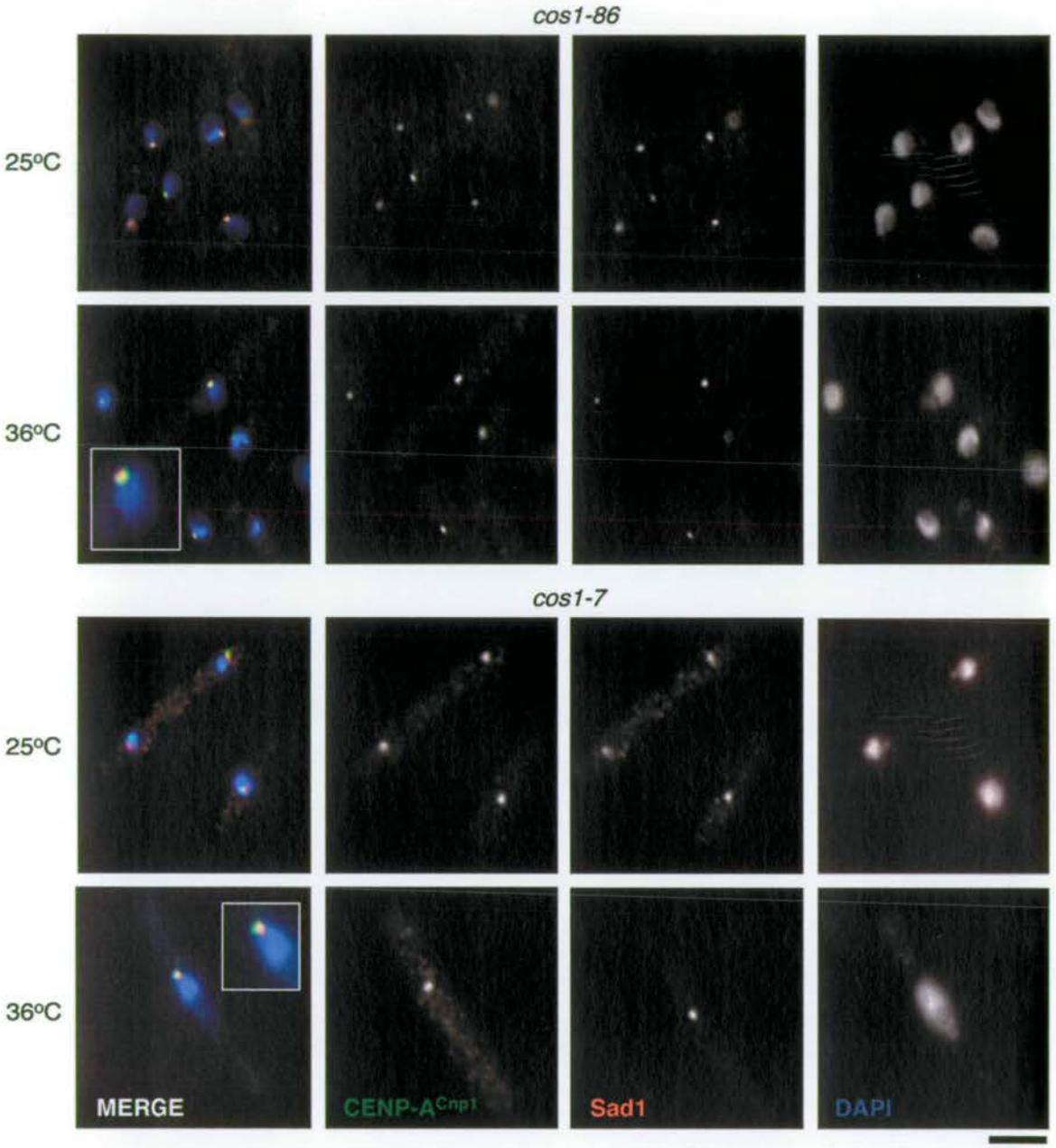


Figure 3-11. CENP-A^{Cnp1} is localised at the centromere in *cos1-86* and *cos1-7* mutants at permissive and restrictive temperature.

Cells were grown at 25°C or for 6 hours at 36°C before fixing and staining with CENP-A^{Cnp1} (green), the spindle pole body marker Sad1 (red) and DNA was stained with DAPI (blue). Centromeres show in increased frequency of declustering at interphase in *cos1-86* and *cos1-7* at 36°C. Inset boxes shown enlarged cells showing declustering. Bar, 5 μm.

(approximately 10%, n=200). Interestingly, *mis6-302* mutants display a similar defect in centromere clustering (Saitoh et al., 1997) and have also been shown to alleviate central core silencing (Partridge et al., 2000). Thus, the clustering of centromere at the nuclear periphery may contribute to silencing or vice versa. Declustering of centromeres in *cos1* mutants may result in cells with a decreased ability to properly position centromeres in interphase and may contribute to aberrant mitotic events downstream.

3.4 Initial analysis of *cos2*, *cos3*, *cos4* and *cos5* mutants

3.4.1 Silencing and segregation defects

As introduced above, *cos2*, *cos4* and *cos5* mutants alleviate silencing at the central core (growth on –arg) and outer repeat domains (growth on –ura and sensitivity to growth on PMG medium supplemented with 5-FOA) (Figure 3-12). Unlike *cos1* mutants, *cos2*, *cos4* and *cos5* mutants were not temperature sensitive and grew as wild type at 36°C. However, like *cos1* mutants, *cos2*, cos4 and *cos5* mutants were sensitive to TBZ treatment. Attempts to clone *cos2*, *cos4* and *cos5* genes by complementation of TBZ^S using Shimoda genomic library were unsuccessful.

cos3-24 was found to alleviate silencing at the central core domain (growth on –arg) and at the telomere (growth on –his). *cos3-24* was not sensitive to the MT destabilizing drug TBZ. DAPI staining of *cos3-24* mutant revealed a high level of segregation defects (approximately 25% cells at 32°C) including lagging chromosomes, hypercondensed chromatin and uneven segregation of DNA (Figure 3-12). Transformation of *cos3-24* with multi-copy plasmids containing Clr4, Chp1 and Swi6, which are required for silencing at telomeres, did not result in complementation of silencing phenotypes.

DISCUSSION

Centromere silencing can be used as an indicator of the structural integrity of chromatin and the structures that assemble on particular regions of chromatin. To date, most mutants that alleviate centromere silencing affect silencing either at the central core or at outer repeat heterochromatin but not both. This chapter describes a screen that utilizes transcriptional silencing to identify mutants that alleviate both central core and outer repeat silencing. The *cos* mutants were isolated from the screen and are discussed below.

Possible roles of genes that are required for central core and outer repeat silencing

cos mutants alleviate silencing at both the central core domain and at outer repeat heterochromatin at centromeres. Cos proteins may encode chromatin-modifying activities

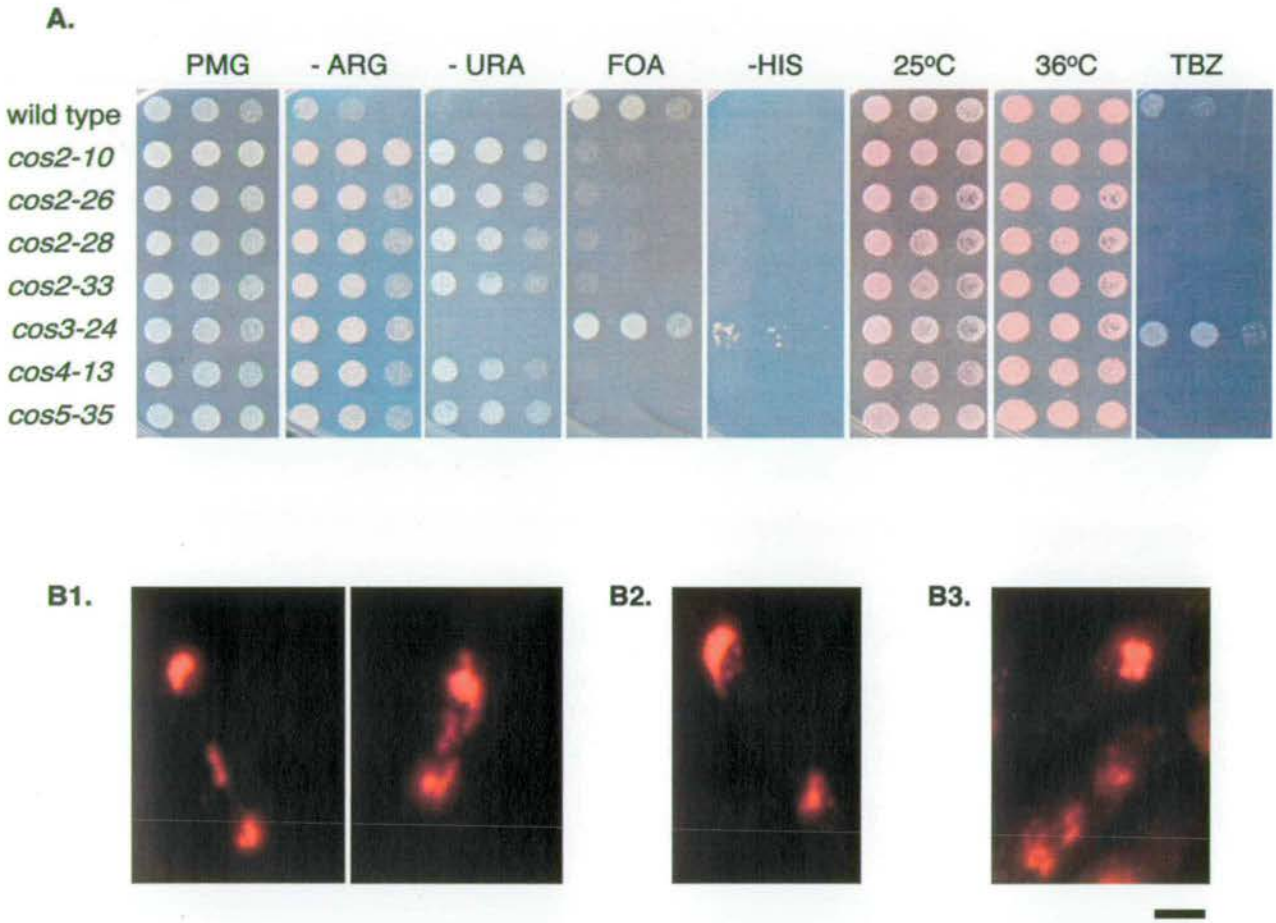


Figure 3-12. Silencing defects and phenotypes of *cos2*, *cos3*, *cos4* and *cos5* mutants.

A. Spotting assay on selective media showing that *cos2, cos4* and *cos5* mutants alleviate central core (grow on medium lacking arginine) and outer repeat silencing (grow on medium lacking uracil and are sensitive to FOA). *cos2, cos4* and *cos5* mutants are sensitive to MT destabilisation by TBZ (10 μg/ml). *cos3-24* alleviates central core and telomeric silencing and is not sensitive to TBZ treatment. *cos2, cos3, cos4* and *cos5* mutants are not temperature sensitive and grow as wild type at 36°C.

B. *cos3-24* shows defects in chromosome segregation. Cells were grown at 32°C, fixed and DAPI stained (red). Segregation defects were detected in 20 % cells analysed (n=100). Types of defects included: lagging chromosomes (B1), unequal segregation of DNA (B2) and hyper-condensed chromatin (B3). Bar, 3 μm.

involved in both the central core and outer repeat chromatin integrity or possess chromatin-remodeling activities that contribute to heterochromatin and kinetochore assembly. Cos-group proteins may need to specifically associate with *cnt* or *otr* or both DNA sequences in order to carry out their function. Alternatively these factors may also play a structural role acting as bridging molecules and may only localize to one domain but affect the structure of the other domain. One hypothesis suggests that although the kinetochore itself forms over the central domain, it is the outer repeat regions that are required to present the central domain in such a way to assemble the kinetochore in the correct configuration to facilitate proper microtubule interactions (Pidoux and Allshire, 2005). Moreover, the outer repeats may define where to place the kinetochore and perhaps the central core is required continually for this process to occur or alternatively only in the initial setting up of the centromere. It has been proposed by Takahashi et al. (1992) that the repeat domains of the centromere interact via looping or coiling of the centromeric DNA and it is also possible that these proteins may contribute to this hypothetical higher order structure at the centromere (see Discussion chapter).

Summary of *cos2*, *cos3*, *cos4* and *cos5* mutant phenotypes

cos2, *cos4* and *cos5* mutants alleviate both central core and outer repeat silencing, are non-temperature sensitive mutations and are sensitive to MT destabilization. *cos3-24* alleviates silencing at the central core domain and at telomeres, is not temperature sensitive and is not sensitive to MT destabilization. The identity of *cos2*, *cos3*, *cos4* and *cos5* genes and their role in chromatin assembly at the centromere remain to be determined. A plasmid containing the *gar2*$^+$ ORF was found to complement the alleviation of silencing at outer repeats and sensitivity to TBZ displayed by all four *cos2* alleles but did not complement the alleviation of central core silencing. *gar2*$^+$ is a nucleolar protein related to nucleolin in vertebrates and is thought to play a role in the assembly of ribosomal components (Leger-Silvestre et al., 1997, Gulli et al., 1995). Sequencing of the *gar2*$^+$ ORF in *cos2* alleles did not reveal any mutations in the *gar2*$^+$ gene, suggesting that *gar2*$^+$ is a suppressor of *cos2* mutants. Further screening using the Shimoda or other genomic libraries available may help clone other *cos* genes. Alternatively, employing genetic mapping techniques may identify the remaining *cos* genes.

cos1$^+$ is allelic to *mcl1*$^+$

cos1 mutants were found to have compromised silencing at central core and outer repeat chromatin, were temperature sensitive and displayed a high frequency of segregation defects. *cos1*$^+$ is allelic to the nuclear mini-chromosome loss gene *mcl1*$^+$. Previous analysis has shown *mcl1*$^+$ to be important for chromosome replication, sister chromatid cohesion and segregation (Williams et al., 2002) and results from this study implies a novel role for Mcl1 in chromatin integrity at the centromere. A very recent study also reported that *mcl1Δ* cells are defective in outer repeat silencing, but central domain silencing was not addressed in

this study (Mamnun et al., 2006).

The extent of the silencing defects at the outer repeats of *cos1* mutants was assessed by a colony colour assay using the *ade6*$^{+}$ reporter gene inserted at *otr*. Mutants such as *clr4Δ*, which is required for the methylation of histone H3 on lysine 9, are completely defective in heterochromatin formation and are white in colour (Ekwall et al., 1996). *cos1* mutants display a partial alleviation of silencing and were found to be pink in colour. Deletion of *swi6Δ* also gives rise pink colonies and this intermediate silencing phenotype may be due to that fact that *swi6Δ* cells still retain normal levels of H3K9me2 and thus other chromodomain proteins such as Chp1 and Chp2. In addition, chromatin IP experiments in *cos1* mutants indicated that despite alleviating silencing at the outer repeats, normal levels of the heterochromatin marker H3K9me2 were present. Other mutants, such as the *csp4*, *csp5*, and *prp10* splicing mutants, alleviate silencing at *otr* and still maintain H3K9me2 on centromeric repeats have been isolated (M. Portoso and E. Bayne, personal communication), however the role of these protein in centromere silencing is as yet unclear. The localization of the chromodomain proteins Swi6, Chp1 and Chp2, which bind to H3K9me2, and contribute further to transcriptional repression at heterochromatin could be determined in *cos1* mutants. Perhaps the improper localization of these chromodomain proteins may help to explain the silencing defects observed in *cos1* mutants. In fact, Mamnun et al. (2006) reported that slight changes in the localization of GFP-Swi6 and Chp1-GFP were detected in *mcl1Δ* cells by fluorescence microscopy (as data not shown). Chromatin IP could be used to localize Swi6, Chp1 and Chp2 to centromeres in cells with defective Mcl1, which is a more sensitive technique to detect subtle effects on localization.

Role of Mcl1 in silent chromatin assembly via replication (Figure 3-13A)

Both Mcl1 and its budding yeast homologue, Ctf4p, have been shown to interact with the DNA replication initiation polymerase α (Polα) (Williams et al., 2005, Miles and Formosa, 1992). In addition, Ctf4p/*CTF4* has been shown to interact both physically and genetically with core components of the replication fork (Hanna et al., 2001, Formosa and Nittis, 1999). It is possible that like Ctf4p, Mcl1 may regulate the association of Polα with replication fork components during S phase. As *mcl1-1* is acutely sensitive to DNA damage in S phase, it is possible that Mcl1 may act more specifically during replication stress (Williams et al., 2005). Thus, Mcl1 may play a role in the replication of topologically constrained regions of the genome, for example sites occupied by cohesin at the centromere. *polα* mutants have been shown to alleviate silencing at the outer repeats of the centromere, at the telomere and at the mating type locus and are defective in Swi6 localisation at these three loci (Nakayama et al., 2001). In addition, Polα has been shown to bind directly to Swi6 *in vitro*. As Mcl1 interacts with Polα, and Polα interacts with Swi6, it is possible that Swi6, Polα and Mcl1 interact in a complex that ensure the features of heterochromatin and the binding of cohesion is propagated and maintained as DNA is replicated. Thus, Mcl1 may exert its

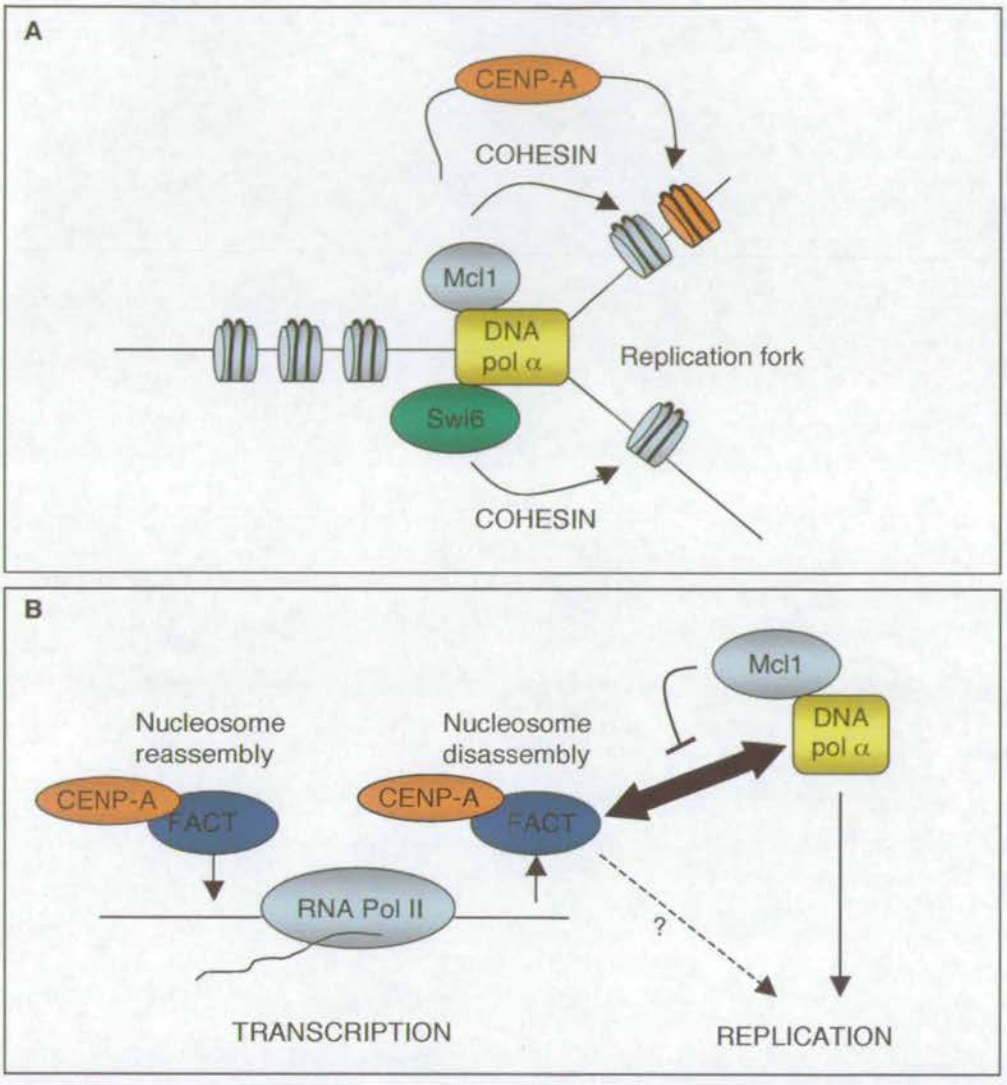


Figure 3-13. A role for Mcl1 in silent chromatin assembly at the centromere.

A. The role of Mcl1 in silent chromatin assembly at centromeres via replication. Mcl1 interacts with the N-terminus of the replication initiation DNA polymerase α (yellow) and Swi6 interacts with the C-terminus of DNA polymerase α. At the replication fork, both Mcl1 and Swi6 may co-operate to ensure that proper sister chromatin cohesion is maintained after passage of the polymerase. Mcl1 may also ensure that CENP-A^{Cnp1} is retained at centromeres at replication.

B. The role of Mcl1 in silent chromatin assembly at centromeres via transcription. As has been shown for its budding yeast homologue Ctf4p, Mcl1 may act to inhibit the interaction between DNA polymerase α and the FACT complex. The FACT (Facilitates Chromatin Transcription) complex consists of two conserved subunits (Cdc68 and Pob3 in fission yeast or FACTp140/p80 in humans) and facilitates nucleosome disassembly and reassembly during the passage of RNA polymerase II at transcription. Mcl1 may act to regulate FACT activity during transcription. It is not known if FACT plays a direct role in replication (indicated by dashed arrow). Both FACT subunits are found in a complex with CENP-A; FACT may facilitate the incorporation of CENP-A^{Cnp1} into chromatin during transcription.

effects on the integrity of central core and outer repeat chromatin through interactions with the DNA replication machinery (see Figure 3-13A). A similar role has been described for the chromatin assembly factor CAF1, which assists in the maintenance of H3K9 methylation and HP1 association with heterochromatin at the replication fork during S phase (Quivy et al., 2004; Sarraf and Stancheva, 2004).

Ctf4p in budding yeast is also required for proper sister chromatid cohesion and it is suggested that Ctf4p may act in association with replication fork components to facilitate the establishment of this robust sister chromatid cohesion (Hanna et al., 2001). *mcl1-1* mutants were also found to exhibit defects in sister chromatin cohesion (Williams et al., 2002). It is possible that Mcl1 acts to maintain levels of Swi6 silent heterochromatin, that are sufficient to recruit Rad21-cohesin and establish proper sister chromatid cohesion either during or after replication. As *mcl1Δ* cells show defective localization of CENP-A^{Cnp1}-GFP (Mamnun et al., 2006), it is also possible that Mcl1 acts to ensure CENP-A^{Cnp1} chromatin is established and maintained at replication. Although no defects in the association of endogenous CENP-A^{Cnp1} with centromeres were detected in the temperature sensitive *cos1/mcl1* alleles described in this chapter, it is possible that the tagged CENP-A ^{Cnp1} is not fully functional and is sensitized to report complex/synthetic defects at centromeres. Also, as *mcl1Δ* cells show extremely poor viability even at the permissive temperature of 25°C, this suggests that the effects on CENP-A^{Cnp1} localization could be indirect and analysis of the temperature sensitive *cos1* point mutations shifted to the restrictive temperature may be a more direct assay of centromere function.

Role of Mcl1 in silent chromatin assembly via transcription (Figure 3-13B)

The budding yeast Mcl1 homologue Ctf4p was also found to inhibit the binding of the essential chromatin-remodeling complex FACT (also known as Cdc68/Pob3 in fission yeast or FACTp140/p80 in humans) to Polα (Wittmeyer and Formosa, 1999). The FACT (Facilitates Chromatin Transcription) complex is required for transcript elongation through nucleosomes by RNA polymerase (Pol II) *in vitro* (Orphanides et al., 1999). One function of FACT is to destabilize the nucleosome so that one H2A-H2B dimer is removed from the nucleosome during the passage of RNA Pol II (Berlotserkovskaya et al., 2003). In addition, FACT facilitates the reassembly of the nucleosome by depositing the H2A-H2B into the disrupted nucleosome in the wake of RNA Pol II transcription (Berlotserkovskaya et al., 2003). This may provide a mechanism by which RNA Pol II can transcribe through chromatin or topologically constrained portions of the genome without disrupting the epigenetic status of nucleosomes. It is possible that in fission yeast, Mcl1 may regulate the chromatin remodeling activity of FACT during transcription. In *cos1* mutants, FACT may gain increased access to central core and outer repeat domains and it is this aberrant chromatin remodeling which results in the alleviation of silencing of these domains and subsequent defects in chromosome segregation. Through its interaction with DNA Polα,

FACT may also play a role in chromatin assembly at replication and Mcl1 may act to regulate the interaction between FACT, DNA Polα and the DNA replication machinery to allow the efficient reassembly of parental nucleosomes after passage of the DNA polymerase at the replication fork.

Mutants defective in the RNAi pathway have been shown to accumulate long unprocessed transcripts from both strands of the outer centromeric repeats as detected in fission yeast (Volpe et al., 2002) and vertebrates (Fukagawa et al., 2004). For this reason, it would be interesting to determine whether centromere transcripts from both outer repeat and central core domains accumulate in *cos1* mutants. It would also be interesting to determine whether FACT plays a role in facilitating this transcription through the specialised chromatin at centromeres. Interestingly, both FACT subunits p140 and p80 were purified as part of a CENP-A complex in HeLa cells in two independent studies (Obuse et al., 2004, Foltz et al., 2006). It is possible that FACT, in concert with Mcl1, provide a more favourable environment for deposition of the CENP-A^{Cnp1} at centromeres and in this manner contributes to silent chromatin formation at centromeres (see Figure 3-13B). It would also be interesting to determine whether the association of CENP-A^{Cnp1} at centromeres is disrupted in FACT mutants and whether FACT, Mcl1 and CENP-A^{Cnp1} are found in a complex in fission yeast.

CHAPTER 4

ANALYSIS OF *sim3* MUTANTS THAT SPECIFICALLY ALLEVIATE CENTRAL CORE SILENCING

INTRODUCTION

The three fission yeast centromeres share the same structural organization and are composed of two distinct chromatin domains: a central domain and outer repeat heterochromatin, which flanks the central domain on either side (Hahnenberger et al., 1991, Takahashi et al., 1992). The histone H3 variant CENP-A^{Cnp1} is assembled in chromatin at the central domain. This organization is similar to metazoan centromeres, where the kinetochore is found embedded in surrounding heterochromatin. Marker genes inserted at sites within fission yeast centromeres are transcriptionally silenced (Allshire et al., 1994, 1995) and this repression is thought to reflect the presence of a functional kinetochore at the central domain and functional heterochromatin that flanks the central domain on either side. The central domain chromatin is distinct from heterochromatin and transcriptional silencing in this domain is weaker and less robust than the repression, which occurs at the outer repeats (Allshire et al., 1994). Outer repeat silent chromatin is heterochromatic and requires the action of the histone methyltransferase *clr4*$^{+}$, which methylates histone H3 on lysine 9 (Rea et al., 2000), providing a binding site for Swi6, the heterochromatin protein 1 (HP1) homologue (Nakayama et al., 2001). The binding of Swi6 to the outer repeats recruits the cohesin subunit Rad21, which ensures the tight physical cohesion of sister centromeres (Bernard et al., 2001, Nonaka et al., 2002). It is now clear that the RNAi machinery is also required to assemble silent heterochromatin (Volpe et al., 2002, 2003, reviewed in Grewal and Moazed, 2003). As a result, mutation or deletion of *clr4*, *swi6* or other heterochromatin or RNAi components results in alleviation of transcriptional silencing at the outer repeats and defects in centromere function (Allshire et al., 1995, Ekwall et al., 1995, 1997, Partridge et al., 2000, Volpe et al., 2002, 2003, Verdel et al., 2004, Motamedi et al., 2004). Thus, there is a correlation between the maintenance of silencing at the centromere and centromere function.

Several mutants have been shown to strongly alleviate silencing specifically at the central core such as the kinetochore components *mis6* (Partridge et al., 2000) and *sim4* (Pidoux et al., 2003). Indeed, mutations in *sim2*$^{+}$ (allelic to *S. pombe cnp1*$^{+}$/CENP-A) itself alleviate central core silencing (Pidoux et al., 2003). Mutants that alleviate central core silencing have no effect on silencing at the outer repeats, with the exception of the *cos* mutants described in chapter 3. In addition, the central core mutants were also found to be defective in the association of CENP-A^{Cnp1} with chromatin and show chromosome segregation defects (Takahashi et al., 2000, Partridge et al., 2000, Pidoux et al., 2003). There is also a correlation

between the alleviation of silencing in the central domain and the location of the cognate protein at the central domain. For example, *mis6* and *sim4* mutants alleviate central core silencing and Mis6 and Sim4 have been shown to be associated with the central core region by chromatin immunoprecipitation (ChIP) (Saitoh et al., 1997, Partridge et al., 2000, Pidoux et al., 2003). Hence, silencing within the central domain is dependent on kinetochore integrity and may be used to assay the presence of functional CENP-A^{Cnp1} chromatin. Central domain chromatin itself is unusual; micrococcal nuclease (MNase) digestion of this domain reveals a smear pattern, rather than the regular nucleosomal ladder pattern typical of most chromatin and is observed in the outer repeat domain (Polizzi and Clarke, 1991; Takahashi et al., 1992). Mutations in central domain associated proteins, such as *mis6* and *sim4*, disrupt this smear pattern and the regular nucleosome ladder pattern is observed (Saitoh et al., 1997, Takahashi et al., 2000, Pidoux et al., 2003). It is likely that it is the presence of CENP-A^{Cnp1} at central domain chromatin that gives rise to the 'unusual' smear pattern as the central domain smear has also been shown to correlate with centromere function on mini-chromosomes (Polizzi and Clarke, 1991, Marschall and Clarke, 1995). However, exceptions to this correlation do exist, for example the *S. pombe* kinetochore mutant *mis12* displays a disrupted central domain digestion pattern, yet CENP-A^{Cnp1} is localized correctly in the *mis12* mutant (Takahashi et al., 2000).

One of the defining features of central domain chromatin in fission yeast, and indeed of centromeres of other species, is the presence of CENP-A^{Cnp1}. However, the mechanism by which CENP-A chromatin is assembled only at the centromere is not fully understood. A number of proteins have been described in fission yeast that contribute to CENP-A^{Cnp1} localisation to centromeres including Mis6, Ams2, Hrp1 and the Mis16-Mis18 complex (Takahashi et al., 2000, Chen et al., 2003, Waldfridsson et al., 2005, Hayashi et al., 2004, Takahashi et al., 2005). Furthermore, the roles of the Mis6 counterpart in vertebrates, CENP-I and human homologues of Mis16, RbAp46/RbAp48, in determining CENP-A localization to centromeres appear to be conserved (Okada et al., 2006, Hayashi et al., 2004). However, additional factors that regulate the establishment and maintenance of CENP-A chromatin at centromeres are likely to exist.

As discussed above, transcriptional silencing at the centromere can be used as tool to monitor kinetochore integrity and the presence of the centromeric histone H3 variant CENP-A. Hence, defective silencing may be indicative of loss of CENP-A^{Cnp1} at centromeres. Previously, a screen exploiting transcriptional silencing at fission yeast centromeres was performed to identify mutations that specifically alleviate silencing within the central domain (Pidoux et al., 2003). As a result the *sim* (silencing in the middle of the centromere) mutants were isolated, which included mutants in the kinetochore protein Sim4, along with mutations in histone fold domains of CENP-A^{Cnp1}. This suggests that silencing within the central domain is dependent on the assembly of CENP-A^{Cnp1} chromatin

and that other *sim* mutants might identify factors more directly involved in the delivery and assembly of CENP-A^{Cnp1} chromatin. The *sim3* mutant was also isolated from the screen. In this chapter, I will discuss the initial characterisation and cloning of *sim3*$^+$ and an investigation into its roles in chromosome segregation, chromatin structure and centromere function.

RESULTS

4.1 *sim3* mutants alleviate silencing at the central core domain

A screen was previously carried out which exploited transcriptional silencing at fission yeast centromeres to isolate mutants that affect centromere function, chromatin structure and chromosome segregation (Pidoux et al., 2003). For this purpose, a strain was constructed with a promoter-compromised *arg3*$^+$ marker gene inserted into *cnt1* (*cnt1:arg3*$^+$) to monitor transcriptional silencing at the central domain as a reporter for centromere function (also described in chapter 3). To monitor silencing at outer repeat heterochromatin this strain was then crossed to a strain with the *ura4*$^+$ gene inserted at *otr2* (Allshire et al., 1994, 1995) and to monitor silencing at the telomere the strain was crossed to a strain with a *his3*$^+$ gene inserted at *tel1* (Nimmo et al., 1998) resulting in the generation of strain FY3027/3033 with the following genotype: *cnt1:arg3*$^+$ *otr2:ura4*$^+$ *cnt3:ade6*$^+$ *tel1L:his3*$^+$. The *ade6*$^+$ gene inserted at *cnt3* proved not to be useful for screening purposes as the *ade6*$^+$ was only weakly repressed in the wild type strain and gave an unacceptable background. From this screen, two alleles of *sim3*$^+$, *sim3-143* and *sim3-205* were identified, which allow strains that harbour a normally silent *arg3*$^+$ within the central domain of centromere 1 to grow faster on plates lacking arginine (Figure 4-1). *sim3* mutants do not affect silencing at outer repeat heterochromatin or at telomeres and were unable to grow on media lacking uracil and histidine, respectively. Both mutants are temperature sensitive for growth at 36°C and appear a dark pink colour on phloxin plates. Three alleles of *sim2* were isolated from the screen and are alleles of *cnp1*, which validates the use of alleviation of transcriptional silencing as a screening approach. *sim2-76* carries a mutation in the histone fold domain of CENP-A^{Cnp1} that is required for CENP-A targeting in human cells (Sullivan et al., 1994) and is also shown to alleviate central core silencing, along with the kinetochore component *mis6-302*. *sim2* and *mis6* do not affect silencing of marker genes inserted at the outer repeats. The *rik1*$^+$ gene is required for outer repeat and telomeric silencing and has no effect on silencing at the central core domain (see Figure 4-1).

4.2 *sim3* mutants are defective in chromosome segregation (Alison Pidoux)

sim3-143 and *sim3-205* were found to be sensitive to the microtubule destabilizing drug TBZ (Figure 4-1). *sim3-143* and *sim3-205* display a variety of abnormal mitotic phenotypes at

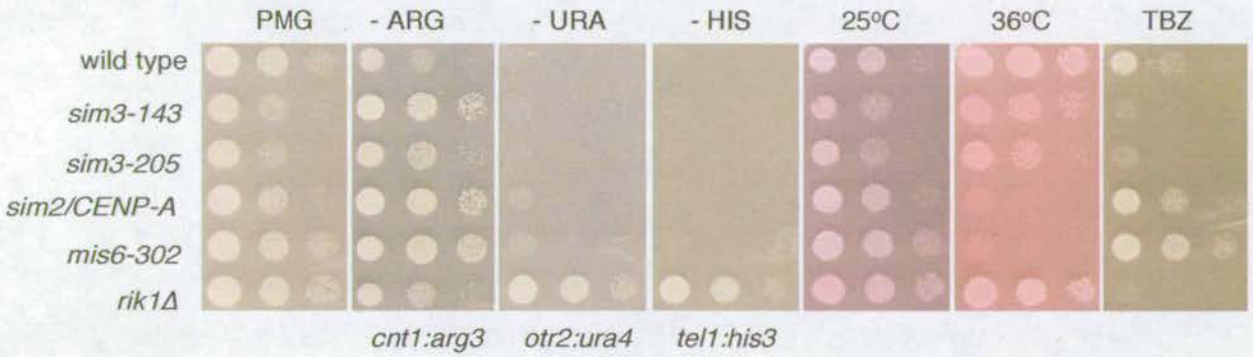
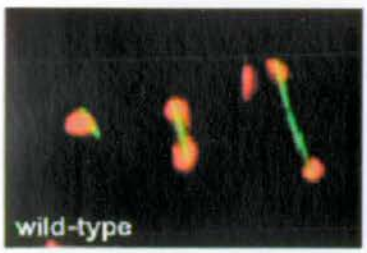


Figure 4-1. *sim3* mutants alleviate central core silencing.

The wild type strain 3027 has *arg3*$^+$ marker gene inserted at central core of centromere 1, a *ura4*$^+$ marker gene inserted at outer repeats of centromere 2 and a *his3*$^+$ marker gene inserted at telomere 1. In wild type cells, silencing of the marker genes is maintained and cells fail to grow on medium lacking arginine, lacking uracil or lacking histidine. *sim3* strains were serially diluted to assay silencing at central core (growth on medium lacking arginine), outer repeat heterochromatin (growth on medium lacking uracil) or telomeres (growth on medium lacking histidine). The first spot contains 5 x 10^3 cells followed by five fold dilutions. Plates were incubated at 25°C for 3 – 7 days or 36°C for 2 days. *sim3-143* and *sim3-205* alleviate central core silencing and do not affect silencing at the outer repeat heterochromatin nor at telomeres. *sim3* mutants are temperature sensitive and form dark pink colonies on medium supplemented with Phloxin B at 36°C. *sim3* mutants are sensitive to MT destabilisation and cannot grow in the presence of TBZ (10 μg/ml). *sim2*$^+$ is allelic to *CENP-A*^{Cnp1}; *sim2-76* and the kinetochore mutant *mis6-302* specifically alleviate silencing at the central core, are temperature sensitive and are not sensitive to TBZ. *rik1Δ* is a heterochromatin mutant that has minimal effect on central core silencing and alleviates silencing at outer repeats and telomeres, is non temperature sensitive and is sensitive to TBZ.

A.



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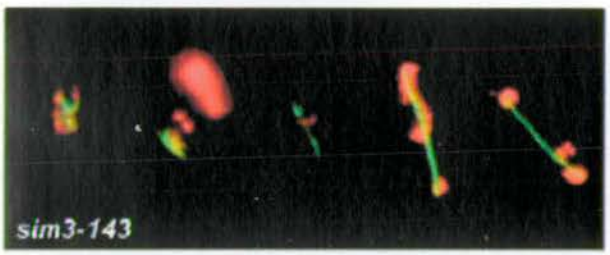

Figure 4-2. *sim3* mutants display defects in chromosome segregation (images from Pidoux et al., 2003).

Wild type and *sim3-143* strain were shifted to 36°C for 6 hours before fixation and immunofluorescence with TAT1 anti-α-tubulin (green) and DAPI staining of DNA (red).

A. Equal chromosome segregation in wild type cells.

B. *sim3-143* mutants show star-shaped spindle (first two cells), hyper-condensed chromatin (middle cell) and lagging chromosomes in anaphase (last two cells).

25°C and 36°C including hypercondensed chromatin, lagging chromosomes on late anaphase spindles and unequal segregation of chromosomes (Pidoux et al., 2003). In addition, star or V shaped spindles were frequently observed in *sim3* mutants (Pidoux et al., 2003). Typical chromosome segregation defects displayed in *sim3-143* are shown in Figure 4-2.

4.3 Cloning of *sim3*$^+$ gene and identification of *sim3* mutations (carried out by Marie Monet)

The gene encoding Sim3 was identified by Marie Monet by complementation with a high copy genomic library (method as described in chapter 3). Transformation of *sim3* with recovered complementing plasmid (pB10D8) was found to restore silencing at the central core domain and resulted in wild type growth on phloxin at 36°C and this was re-confirmed by a growth assay of *sim3* mutants transformed with plasmid on selective media (Figure 4-3). Sequencing of inserts of complementing plasmids, using universal sequencing primers that anneal to the multiple cloning site of pAL-KS plasmid, showed that they contain the open reading frame (ORF) SPCC577.15c (Marie Monet). Sequencing of this ORF in *sim3-143* and *sim3-205* revealed the presence of the mis-sense mutations G81E and E207K, respectively, confirming it to be defective in these mutants (Marie Monet) (see Figure 4-4).

4.4 *sim3*$^+$ is an essential gene that shows homology to the histone chaperones N1/N2 and NASP

Sim3 contains four tetratricopeptide repeats (TPR) which comprise almost the entire Sim3 protein and BLAST searches reveal similarity to human nuclear autoantigenic sperm protein (NASP) and *Xenopus* N1/N2, both of which have been shown to be histone binding proteins (Kleinschmidt et al., 1985; Kleinschmidt et al., 1990, Richardson et al., 2000), (Figure 4-4). Sim3 shows approximately 32% identity to human NASP (somatic), 33% identity to *Xenopus laevis* N1/N2 and 24% identity to *Drosophila melanogaster* DmNASP. No related protein is encoded by the genome of *Saccharomyces cerevisiae*, although similar proteins are present in other fungi including *Candida albicans*. N1/N2 is complexed with stored histone H3 and H4 in *Xenopus laevis* oocytes (Kleinschmidt et al., 1985) while NASP has been shown to co-purify from HeLa cells with both histone H3.1 and the H3 replacement variant H3.3 (Tagami et al., 2004). In addition NASP has been shown to bind the linker histone H1 (Richardson et al., 2000) and has been shown to be essential for cell proliferation in homozygous NASP$^{-/-}$ null mice (Richardson et al., 2006). Deletion of *sim3*$^+$ was carried out by the insertion of the *ura4*$^+$ marker gene at the *sim3*$^+$ ORF by homologous recombination in a diploid strain. Subsequent sporulation and analysis of spores by both random spore analysis and tetrad dissection demonstrated that, like mammalian NASP, the *sim3*$^+$ gene is essential for viability (data from Marie Monet). The Sim3 mutations occur in conserved

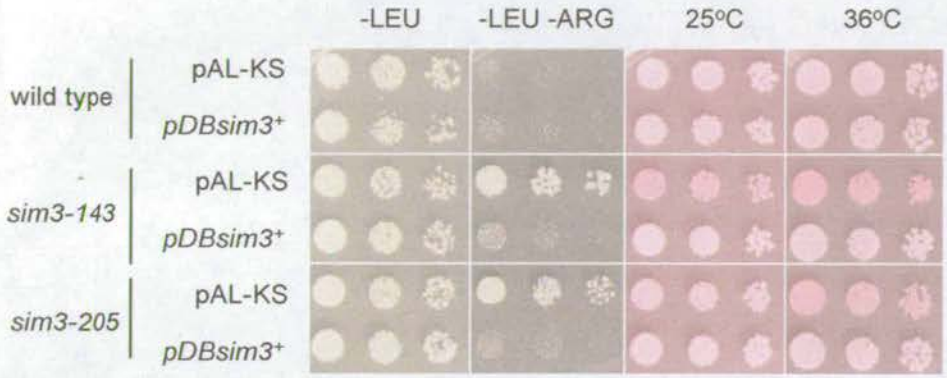

Figure 4-3. *sim3* mutants are complemented by plasmid bearing the *sim3*$^+$ ORF (SPCC577.15c).

Plasmid pB10D8, which rescued the temperature sensitivity of *sim3* (Marie Monet), was re-transformed into *sim3* mutant alleles, along with a control empty *LEU2*$^+$ plasmid pAL-KS. In wild type cells (3027), an *arg3*$^+$ gene inserted at the central core silenced and form tiny colonies on plates lacking arginine. *sim3* mutants alleviate silencing at the central core and grow on medium lacking arginine. pB10D8 containing *sim3*$^+$ ORF reimposes silencing at *cnt1:arg3*$^+$ and complements the temperature sensitive phenotype of *sim3* mutants as assayed by growth of light pink colonies on Phloxin B at 36°C.

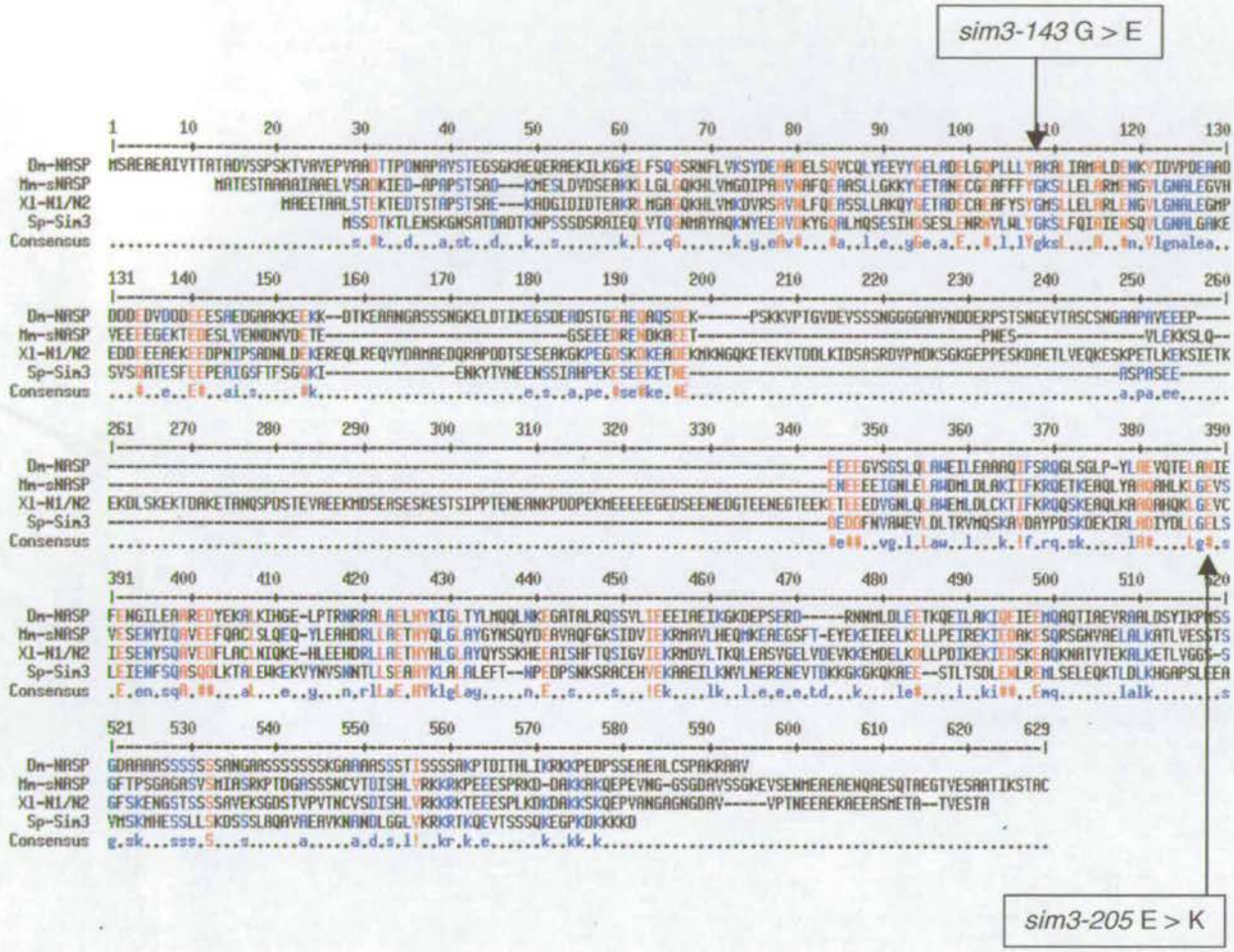

Figure 4-4. Sim3 show homology to *Xenopus laevis* N1/N2 and mammalian NASP.

BLAST alignment showing similarity of Sim3 to the histone binding proteins *Xenopus laevis* N1/N2 and human nuclear autoantigenic sperm protein (NASP). Sim3 shows 32% identity to mammalian NASP, 33% identity to *Xenopus laevis* N1/N2 and 24% identity to *Drosophila melanogaster* DmNASP. Sequencing of SPCC577.15c ORF in *sim3-143* and *sim3-205* revealed the presence of the mis-sense mutations G81E and E207K, respectively (carried out by Marie Monet). Sim3 point mutations G81E and E270K mutations are in highly conserved residues close to or within the tetratricopeptide (TPR) repeats. *Dm, Drosophila melanogaster, Mm, Mus musculus, Xl, Xenopus laevis, Sp, Schizosaccharomyces pombe.*

residues in evolutionarily conserved blocks close to or within the TPR repeats (Figure 4-4). Multiple alignments suggest that *S. pombe* Sim3 and other fungal homologues are diverged from vertebrate NASP/N1/N2 (not shown).

4.5 Production of anti-Sim3 antibodies

In order to investigate further the role of Sim3 in the centromeric chromatin and chromosome segregation, antibodies against the entire Sim3 protein were raised. Full-length recombinant GST-Sim3 (70 kDa) was produced in bacteria (Figure 4-5) and GST-Sim3 fusion protein was used for immunisation and antibody production in two rabbits (R6376, R6388). To reduce background staining, anti-Sim3 serum was affinity purified against recombinant Sim3 protein cleaved away from the GST affinity tag, as the serum may contain antibodies that recognize GST alone. Approximately 95 % of GST-Sim3 was cleaved after 1 hour incubation with thrombin (Figure 4-5) and this cleaved Sim3 was immobilized on nitrocellulose membrane for subsequent affinity purification of the serum. Affinity purified anti-Sim3 was found to specifically recognise a 55 kDa band (predicted molecular weight of Sim3 is 43 kDa) protein by western analysis. This band increased in intensity when Sim3 was over-expressed. Anti-Sim3 also recognised Sim3-GFP protein 80 kDa (predicted molecular weight of Sim3-GFP is 70 kDa), in a strain where Sim3 is tagged with GFP at its endogenous locus and the band for wild type Sim3 was no longer seen (Figure 4-6A). This indicates that anti-Sim3 specifically recognises the Sim3 protein.

Affinity purified anti-Sim3 antibodies were next used to determine the localization of the Sim3 protein on fixed wild type cells by immuno-fluorescence microscopy. Affinity purified anti-Sim3 staining from both rabbits showed a bright nuclear signal, which correlates with the published nuclear localization of NASP and N1/N2. This signal was not detected when cells were stained with pre-immune serum only (Figure 4-6B).

4.6 Sim3 mutant protein is stable at restrictive temperature

In order to determine whether the Sim3 protein made in the temperature sensitive mutants *sim3-143* and *sim4-205* was stable at restrictive temperature, extracts were made from wild type and *sim3* mutants at permissive (25°C) and restrictive temperature (6 hours at 36°C) and were analysed by western blotting with anti-Sim3. Sim3 mutant protein was still detectable and stable in *sim3* mutants even at 36°C (Figure 4-7). This indicates that the defect in *sim3* mutants is not due to instability/degradation of temperature sensitive proteins made in *sim3-143* or *sim3-205* mutants. This implies that the increased temperature causes a misfolding/unfolding of the protein or a structural change rendering it incapable of carrying out its function.

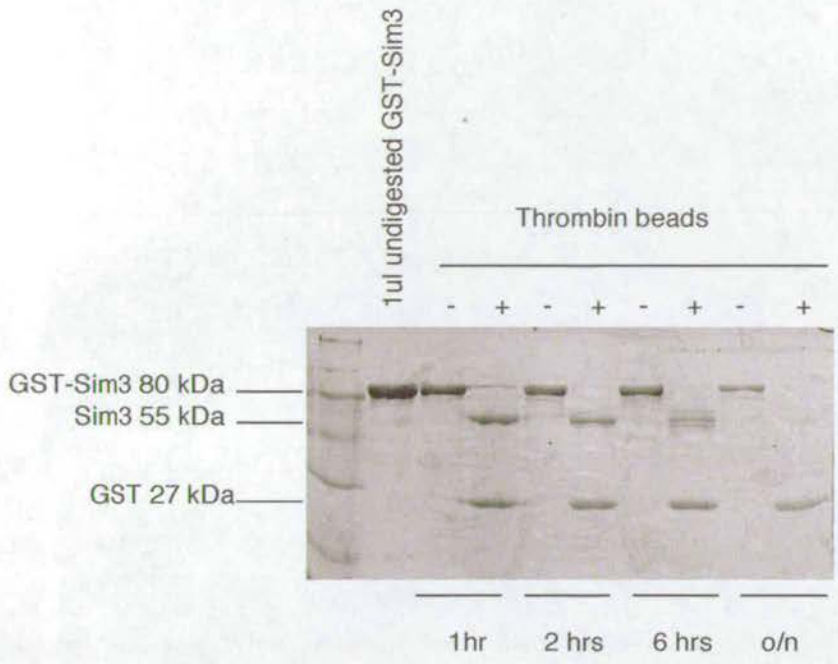

Figure 4-5. Purification of GST-Sim3 in *E. coli* and cleavage of GST-Sim3 by thrombin.

Recombinant GST-Sim3 was produced in *E. coli*. GST-Sim3 was incubated with thrombin beads to cleave GST for 1 hour to overnight at room temperature, samples were run on an SDS-PAGE gel and coomassie stained. GST-Sim3 runs at approximately 80 kDa, higher that its calculated molecular weight of 70 kDa. Incubation with thrombin beads results in removal of the GST tag (27 kDa) and cleaved Sim3, which runs at approximately 55 kDa is visible on the gel (predicted molecular weight is 43 kDa). Optimal cleavage conditions were achieved after 1 hour incubation with thrombin at room temperature.

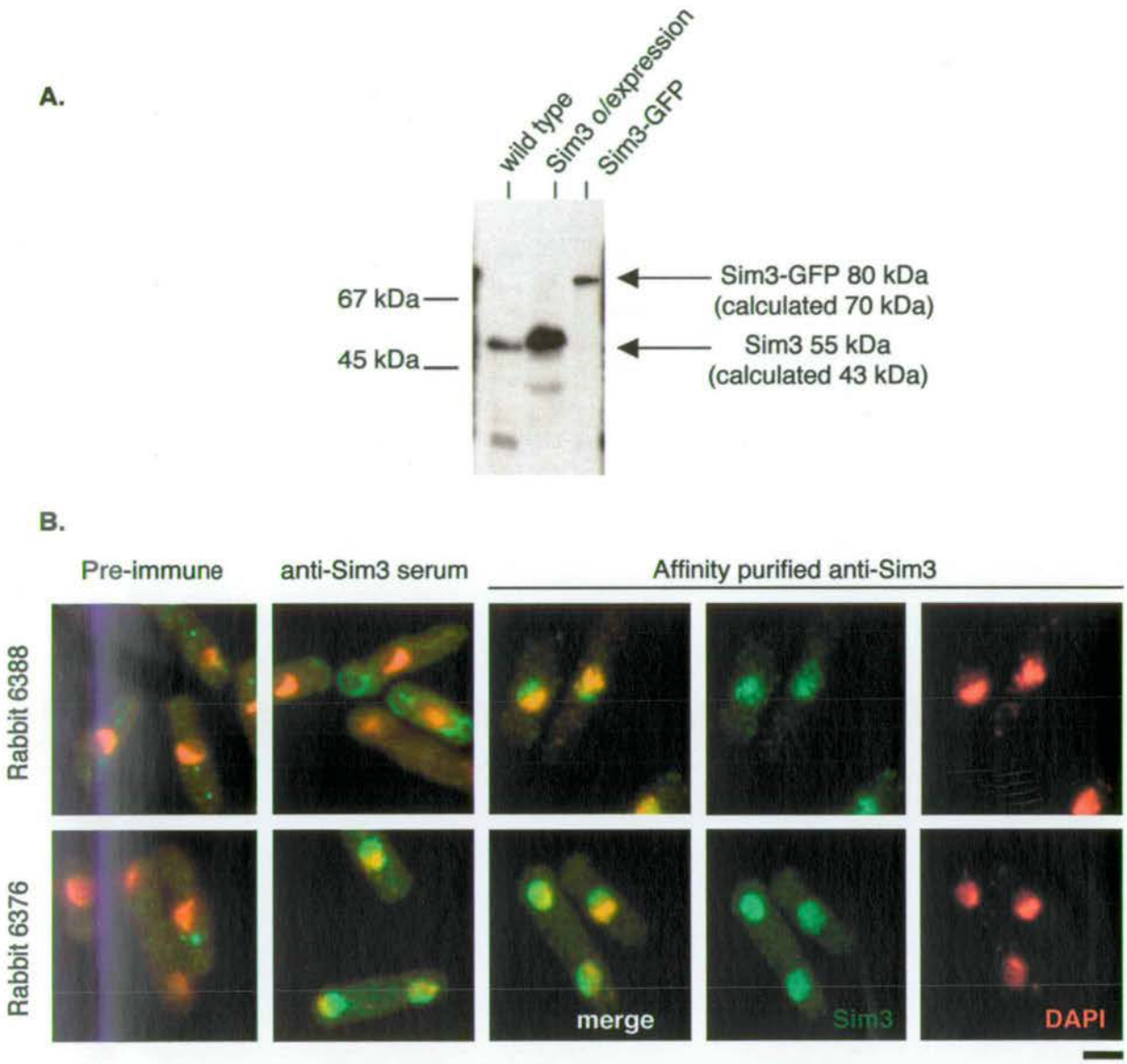

Figure 4-6. Affinity purified anti-Sim3 is specifically recognises Sim3 by western blot and shows a nuclear signal by immunofluorescent staining.

A. Affinity purified anti-Sim3 recogises Sim3 by western blot of wild type extracts (43.9 kDa, runs at 55 kDa) and this band increased in intensity on Sim3 over-expression in strains transformed with genomic high copy plasmid encoding *sim3*$^{+}$ ORF. Anti-Sim3 also recognises Sim3-GFP (70 kDa, runs at 80 kDa) and band for endogenous Sim3 disappeared as Sim3 is GFP tagged at its endogenous locus and is the only copy of Sim3 in the cell.

B. Wild type strains were stained with pre-immune rabbit serum, anti-Sim3 serum and affinity purified anti-Sim3 and viewed by immunofluorescence. GST-Sim3 was used to immunize rabbit 6376 and rabbit 6388. Antibodies from both rabbits showed a nuclear localisation pattern on affinity purification. Pre-immune serum showed only background staining. Bar 3 μm.

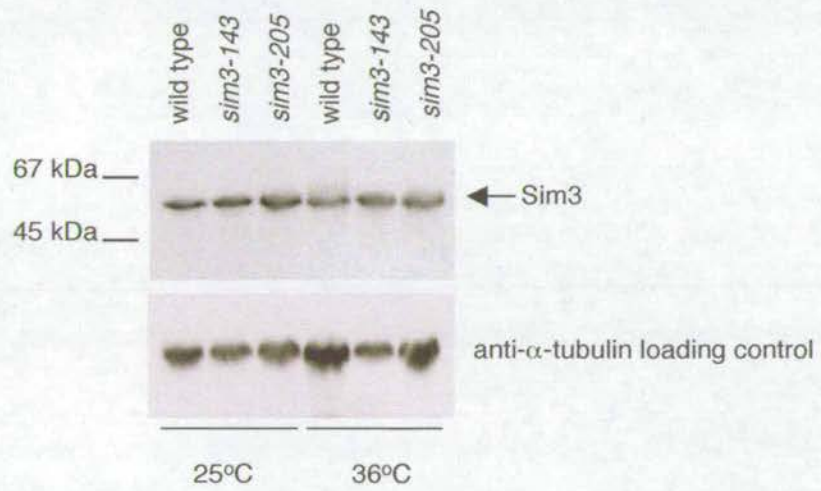

Figure 4-7. Sim3 mutant protein is stable at 36°C by anti-Sim3 western analysis.

Extracts were prepared from wild type and *sim3* mutants at permissive temperature of 25°C or were shifted to restrictive temperature of 36°C for 6 hours. Sim3 protein is detectable in wild type and mutants at both temperatures. Proteins were separated by SDS-PAGE, transferred to nitrocellulose and incubated with anti-Sim3 and anti-α-tubulin (55 kDa) was used as a loading control.

4.7 Sim3 localises to the nucleus throughout the cell cycle

Other proteins involved in kinetochore function and CENP-A^{Cnp1} association with the central kineochore domain, have been shown to alleviate central core silencing and have been found to localise at centromeres and associate with the central domain themselves, such as Mis6 and Sim4 (Pidoux et al., 2003, Partridge et al., 2000), as described above. For example it is possible that Sim3 only localizes at centromeres during mitosis. To address this, anti-Sim3 antibodies were used to stain wild type cells along with anti-α-tubulin as a marker of mitotic stages of the cell cycle. As reported for NASP and N1/N2 (O'Rand et al., 1992, Richardson et al., 2000, Kleinschmidt et al., 1988), Sim3 localised to the nucleoplasm, staining both the nucleus and nucleolus throughout the cell cycle (Figure 4-8). Sim3 appears to have an uneven and punctate distribution. In addition, the localisation of mutant Sim3 protein was determined by staining *sim3* mutants with anti-Sim3 at both permissive and restrictive temperature. Sim3 mutant protein localized to the nucleus as wild type at 25°C but there appeared to be more Sim3 signal in the cytoplasm of *sim3* mutants at 36°C (Figure 4-9). This increase in cytoplasmic staining may be due to an accumulation of the mutant protein in the cytoplasm or maybe an artifact of fixation of mutants at restrictive temperature.

4.8 Tagged Sim3-GFP protein is functional

To directly visualize Sim3 in living cells, the *sim3*$^+$ ORF was tagged with GFP under the control of its native promoter at its endogenous locus (carried out by Marie Monet). Briefly, the 3′ terminal 650 bp of the *sim3*$^+$ ORF was cloned as a *KpnI-SalI* fragment into the pDM84 plasmid, containing the GFP gene and a *his3*$^+$ marker gene for selection. The resulting construct was linearised by a *PstI* digestion and transformed into a wild type *his*$^-$ strain (2221 and 1645). *his*$^+$ transformants were selected and integration of GFP at C terminus of *sim3*$^+$ ORF was checked by PCR using a forward primer within the *sim3*$^+$ ORF and a reverse primer within the GFP gene. Mutant *sim3-143* (*sim3-143*^{ts}-GFP) was also tagged using the same plasmid. It was not possible to tag *sim3-205* using the same strategy as the mutation is in the 3′ terminal 650 bp of the *sim3* ORF and is too close to the 3′ end to allow tagging by recombination (Figure 4-10).

Both *sim3*$^+$-GFP and *sim3-143*^{ts}-GFP tagged strains were constructed in a strain which had *arg3*$^+$ gene inserted in the central domain of centromere 1. The tagged strains were subsequently tested for defects in central core silencing by serial dilution growth assays onto media lacking arginine. The *sim3*$^+$-GFP tagged strain maintains silencing at *cnt1:arg3*$^+$, whereas the *sim3-143*^{ts}-GFP tagged strain alleviates silencing of *arg3*$^+$ at the central core domain. To test the viability of the GFP-tagged strains cells were serially diluted onto media supplemented with phloxin, to assay for temperature sensitivity. *sim3*$^+$-GFP can

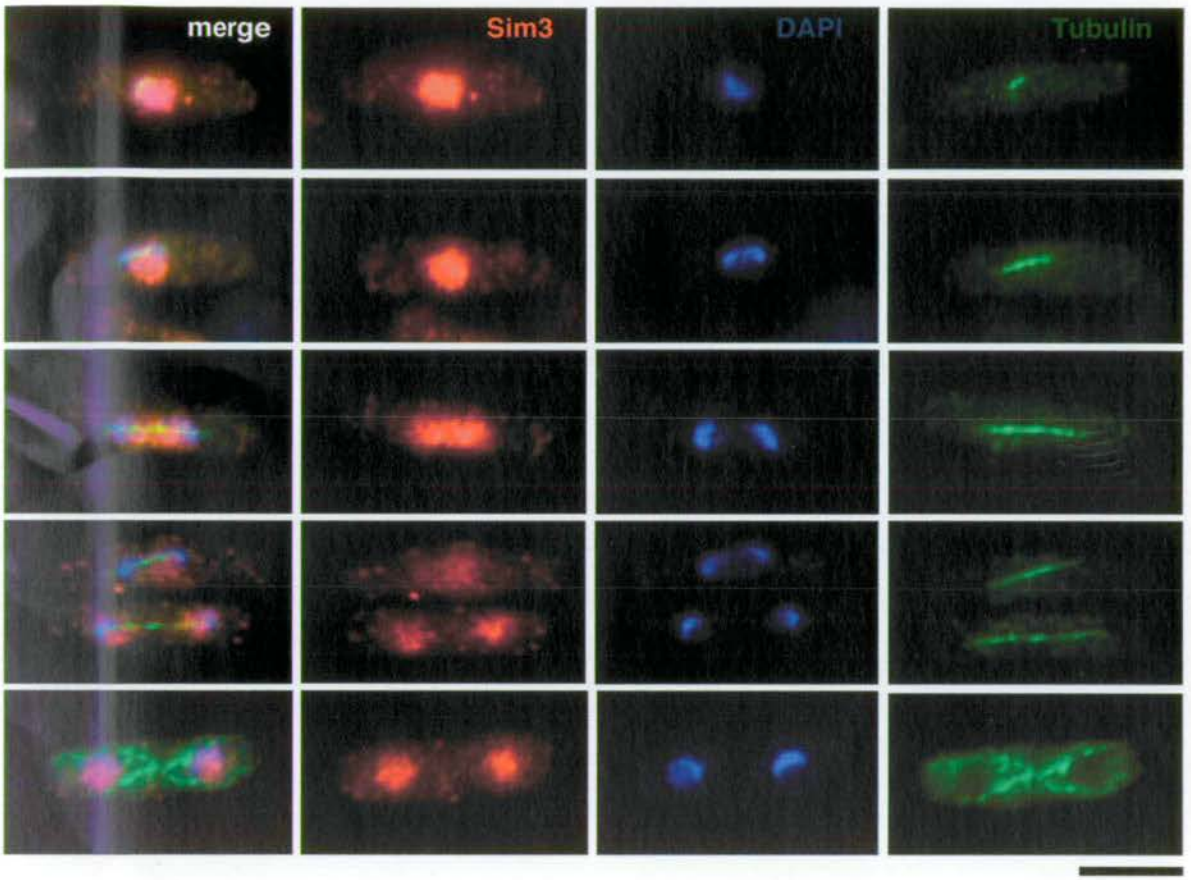

Figure 4-8. Sim3 is localised to the nucleus throughout mitosis.

Wild type cells were grown to log phase, fixed and stained with anti-Sim3 (red), anti-α-tubulin (green) and DNA was stained in DAPI (blue). A merged image is also shown. Bar, 5 μm.

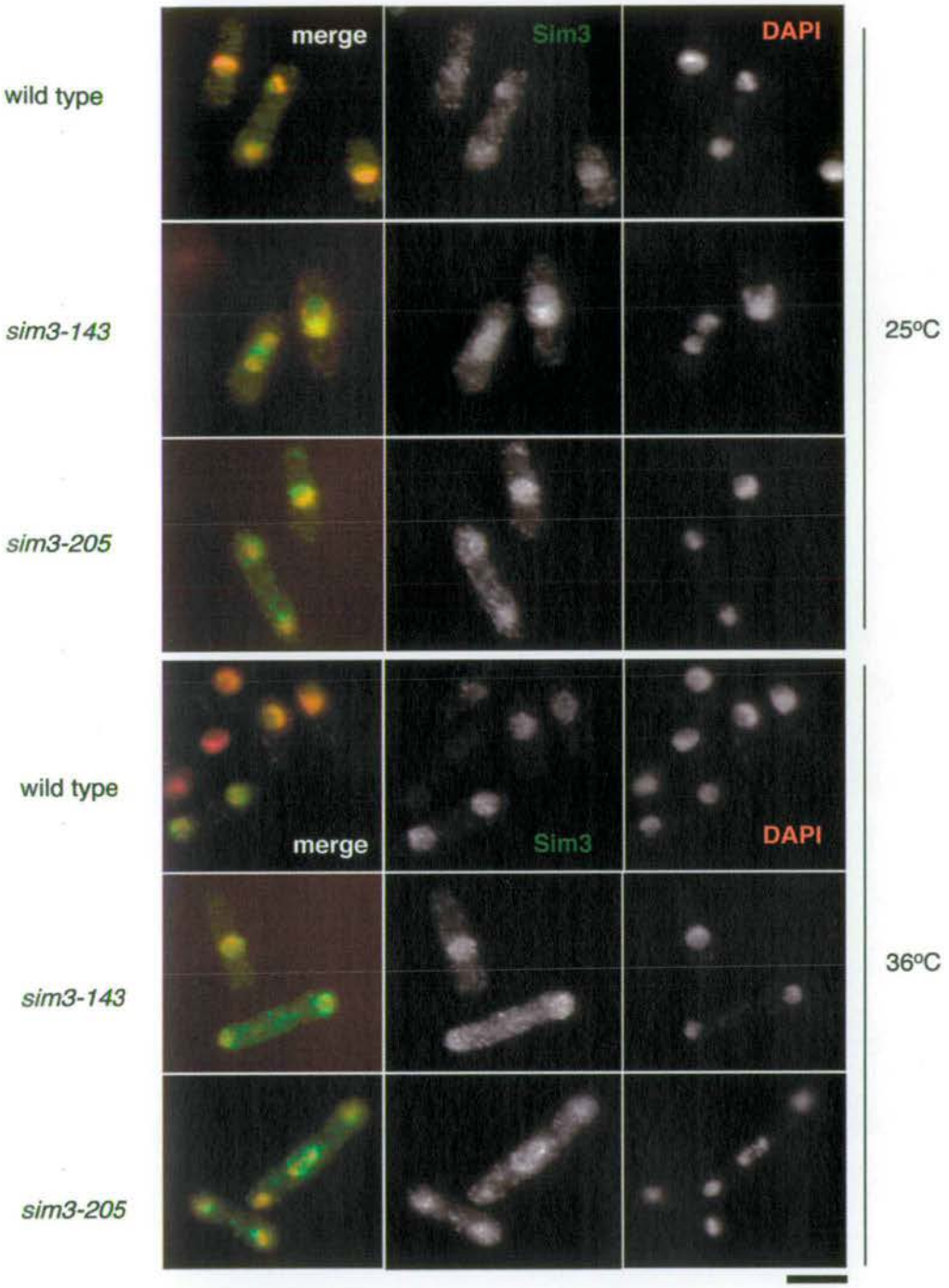

Figure 4-9. Localisation of Sim3 in *sim3* mutants at permissive and restrictive temperature.

Wild type and *sim3* mutants were either grown at 25°C or were shifted to restrictive temperature of 36°C for 6 hours and were stained with anti-Sim3. Sim3 (green) is enriched in the nucleus in wild type and *sim3* mutants at 25°C. An increase in Sim3 signal from the cytoplasm was observed in *sim3* mutants stained with anti-Sim3 at the restrictive temperature. Bar, 5 μm.

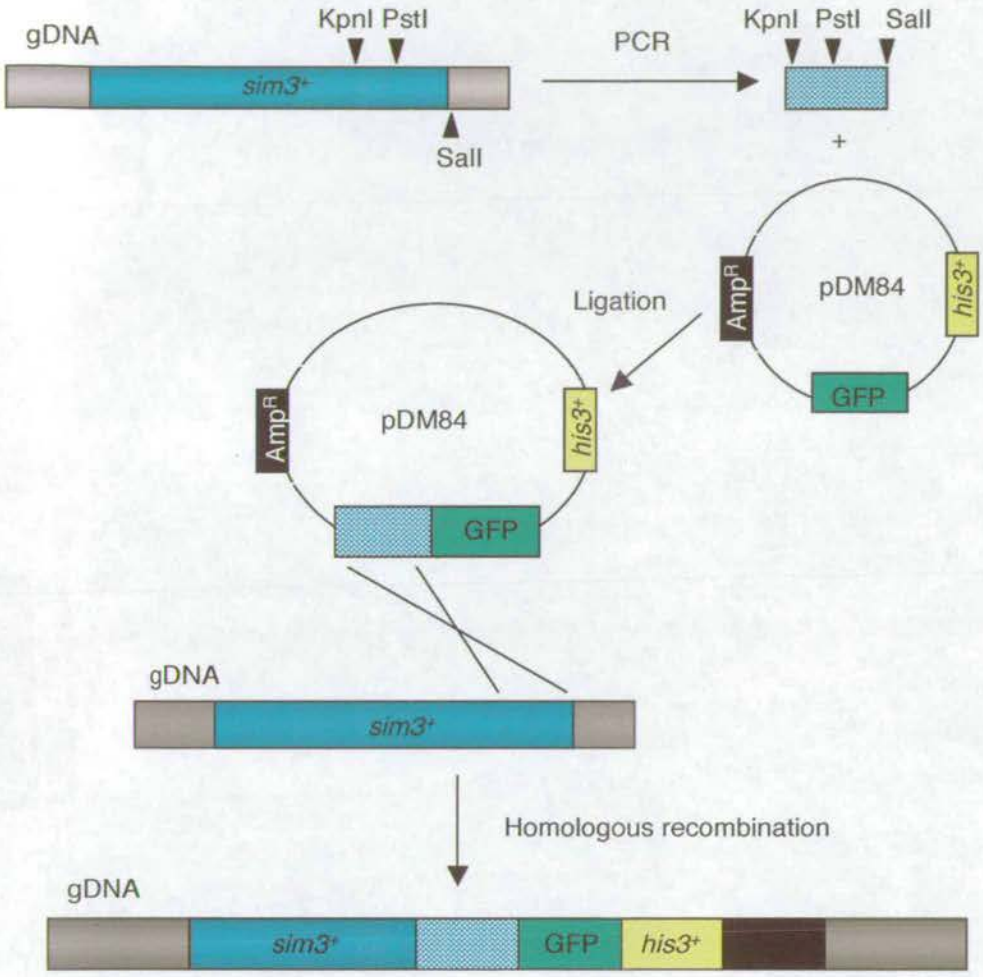

Figure 4-10. Tagging of Sim3 at the C terminus under its own promoter with GFP.

The 3' terminus of the *sim3*+ ORF was cloned as a *KpnI-SalI* fragment into the pDM84 plasmid containing the GFP gene, a *his3*+ marker and an Ampicillin resistance gene for selection. Plasmid was then linearised by *PstI* digest and was integrated at *sim3*+ ORF by homologous recombination after transformation. Mutant *sim3-143* was tagged using the same strategy.

grow at 36°C as wild type control strain and *sim3-143^{ts}*-GFP is temperature sensitive, as is the *sim3-143* mutant (Figure 4-11A).

4.9 Tagged Sim3-GFP is localised to the nucleus

To further investigate the localization of Sim3, strains expressing wild type and mutant Sim3 tagged with GFP were analysed by live imaging. Sim3-GFP was found to be evenly distributed throughout the nucleus. This nuclear localization was reduced and there appeared to be an increase in GFP signal from the cytoplasm in cells expressing *sim3-143^{ts}*-GFP strain (Figure 4-11B).

To assess whether the punctate staining pattern that was observed with anti-Sim3 immunofluorescence was an artifact of fixation/staining, *sim3^{+}*-GFP strains were fixed and stained with anti-Sim3 or anti-GFP antibodies or fixed only and viewed. In all cases where cells were fixed, a punctate pattern was observed (not shown). As this differs from the even distribution of Sim3 observed from analysis of live cells, this uneven localization pattern is very likely to be an artifact of fixation.

Examination of fixed *sim3^{+}*-GFP cells stained with anti-GFP antibody showed that 100% of cells had a nuclear localization at 25°C and 36°C. *sim3-143^{ts}*-GFP cells showed either of two patterns of localization at 36°C: an increased signal in the cytoplasm plus an enrichment of signal in the nucleus (74 % cells) or an equivalent signal from both cytoplasm and nucleus (26% cells), (Figure 4-12). This result correlates with the increased cytoplasmic staining that was observed in *sim3* mutants stained with anti-Sim3 at restrictive temperature as discussed above. These results suggest that either mutant Sim3 protein maybe delocalized from the nucleus or cannot gain access to the nucleus or cannot be retained in the nucleus. In addition, it is also possible that mutant non-functional Sim3 protein accumulates in the cytoplasm where it is targeted for degradation, although no accumulation of Sim3 protein was detected in *sim3* mutants by western analysis.

4.10 Sim3 does not appear to be enriched at centromeres

Kinetochore mutants such as *mal2*, *mis6* and *sim4* alleviate central core silencing and Mal2, Mis6 and Sim4 have been shown to localize cytologically to the kinetochore and specifically bind to the central core region (Jin et al., 2002, Partridge et al., 2000, Saitoh et al., 1996, Pidoux et al., 2003). Thus, there is a correlation between the loss of silencing at a particular region of chromatin and the association of the protein with that region. As *sim3* mutants alleviate silencing, it is possible that Sim3 may function at the centromere and need to associate with the centromere at a certain point. To investigate this possibility, anti-Sim3 staining was firstly carried out on wild type cells, which were co-stained with anti-CENP-

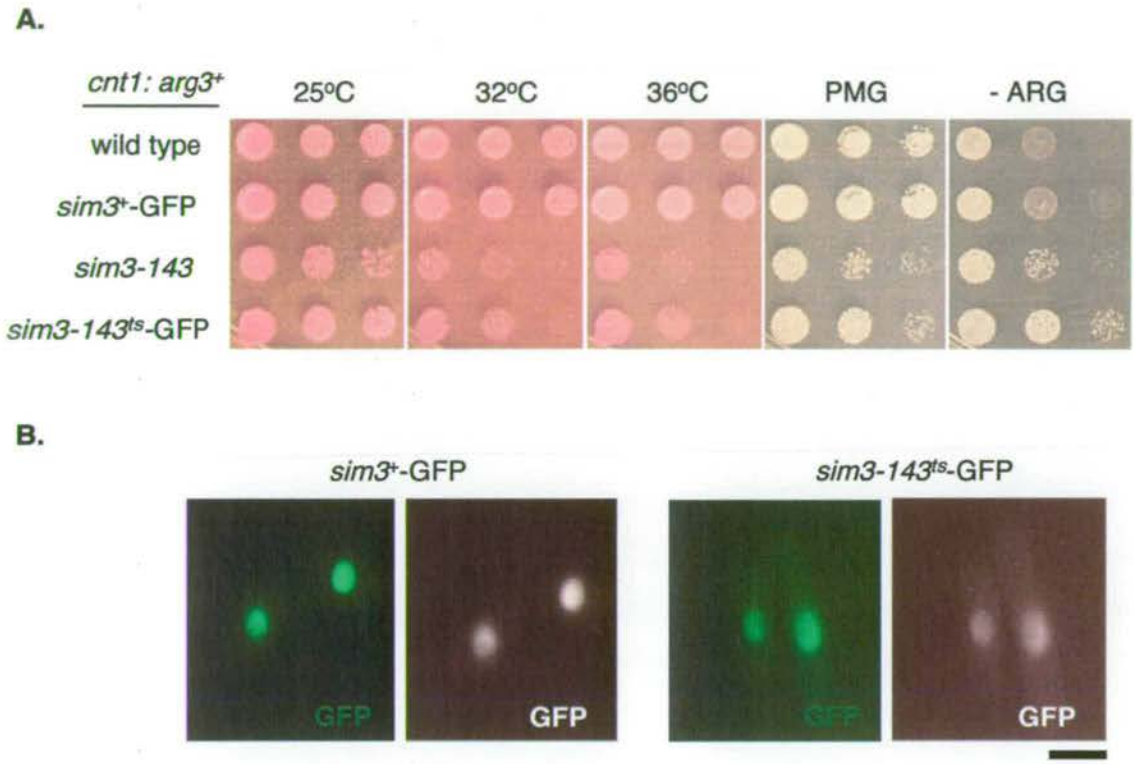

Figure 4-11. Phenotype and localisation of *sim3*⁺ tagged with GFP and *sim3-143*ᵗˢ tagged with GFP.

A. Wild type, *sim3-143, sim3*⁺-GFP and *sim3-143*ᵗˢ-GFP tagged strains (with an *arg3*⁺ gene inserted at the central core) were spotted onto medium lacking arginine to test for silencing defects and onto Phloxin B at 25°C, 32°C and 36°C to test for temperature sensitivity. *sim3*-GFP strain was wild type with respect to silencing and ts. *sim3-143*ᵗˢ-GFP showed alleviation of central core silencing and was ts, as was *sim3-143*.

B. Live imaging of Sim3-GFP showed an even nuclear distribution. Live imaging of Sim3-143ᵗˢ-GFP showed a nuclear distribution with an increase in GFP signal from the cytoplasm. Bar, 3 μm.

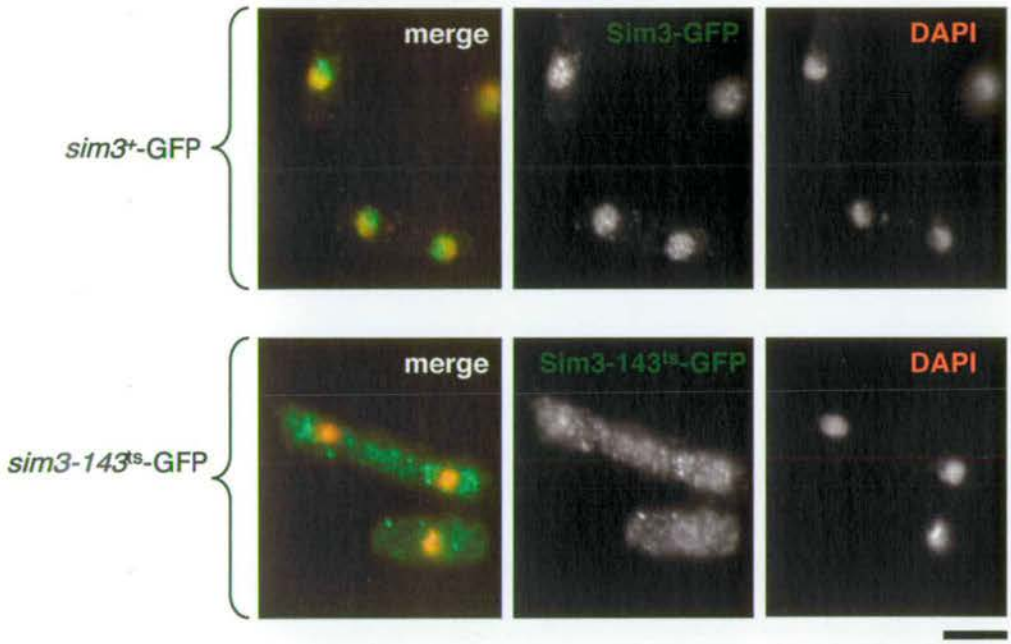

Figure 4-12. *sim3*$^{+}$-GFP and *sim3-143*^{ts}-GFP strains stained with anti-GFP at 36°C.

Sim3-GFP shows a punctate nuclear pattern when fixed and stained with anti-GFP antibodies. Live imaging of Sim3-GFP expressing cells showed an even nuclear distrubtion, suggesting the punctate pattern is an artifact of fixation.

Sim3-143^{ts}-GFP shows a punctate pattern which is no longer enriched in the nucleus and shows increased cytoplasmic signal when stained with anti-GFP at 36°C. A similar pattern was observed for *sim3* mutants stained with anti-Sim3 at 36°C (see previous Figure 4-9). Bar, 5 μm.

A^{Cnp1} to mark centromeres. Though Sim3 staining often appeared punctate in the nucleoplasm, these Sim3 spots (possibly an artifact of fixation) did not colocalise with CENP-A^{Cnp1} spots (Figure 4-13A). Secondly, in order to visualize the three individual fission yeast chromosomes, which are not normally visible in wild type cells, the *nda3-KM311* mutant was utilized. *nda3-KM311* mutants are cold sensitive β-tubulin mutants, which are arrested by growing overnight at 18°C and which undergo multiple rounds of DNA condensation, where DNA becomes hypercondensed and individual chromosomes are visible (Hiraoka et al., 1984). After the arrest was induced, *nda3* cells were fixed and co-stained with anti-Sim3 and anti-CENP-A^{Cnp1}. This analysis shows that Sim3 appears to fill the entire nucleus and is not tightly associated with chromatin nor is it specifically associated with the centromeric CENP-A^{Cnp1} spot (Figure 4-13B).

To determine if Sim3 was associated with specific centromeric sequences, anti-GFP ChIP was carried out in the *sim3*$^{+}$-GFP tagged strain. Analysis of immunoprecipitated DNA indicated that Sim3-GFP was not enriched at either central core (*cnt*) or outer repeat sequences (*otr*). The kinetochore component Mal2-GFP was used as a positive control and was found to be enriched at *cnt* sequences, as previously shown (Jin et al., 2002), (Figure 4-14). These results of immuno-fluorescent staining and ChIP suggest that unlike other proteins isolated from the *sim* screen, Sim3 is not stably associated with the centromere. Sim3 may however be transiently associated with the centromeres at an undetectable levels or at a particular stage of the cell cycle.

4.11 *sim3* interacts genetically with mutations in other kinetochore proteins/genes (Pidoux et al., 2003)

Genetic interactions between *sim3* and other kinetochore mutants were assessed in Pidoux et al. (2003). Over-expression of CENP-A^{Cnp1} and Sim4, but not Mis6, suppressed the temperature sensitivity of *sim3-143*. *sim3-143* was synthetically lethal in combination with *sim2/CENP-A*^{cnp1} or *sim4* or *mal2* mutations, whereas *sim3-143 mis6-302* double mutants showed no growth impairment compared with single mutants. *sim3-143* showed a synthetic interaction with the spindle checkpoint mutants, *bub1* and *bub3*, where double mutants were highly growth impaired. In contrast, no synthetic lethality was observed between *sim2/CENP-A*^{cnp1} or *sim4* or *mis6-302* mutants and the spindle checkpoint mutants *bub1*, *bub3*, *mad2* and *mad3*. These genetic interactions between Sim3 and other kinetochore components are indicative of a role for Sim3 at the kinetochore/centromere.

4.12 Analysis of Sim3 over-expression

Over-expression of a protein often results in the suppression of the phenotype of mutants in the same pathway or complexes. To gain insight into the possible functions of Sim3 at the

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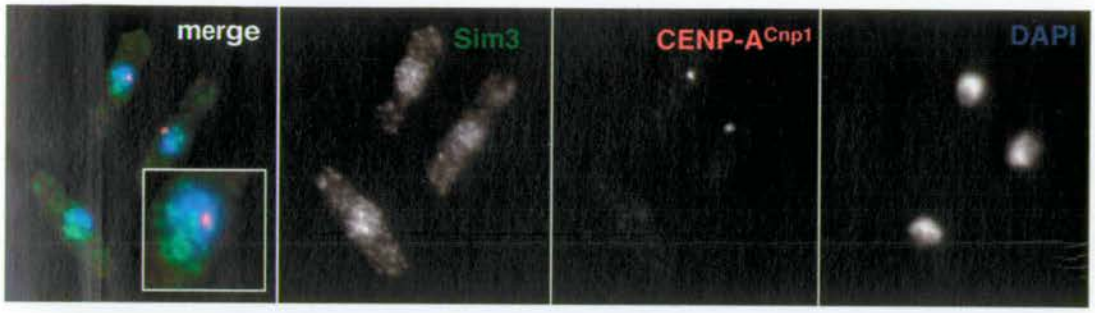


B.

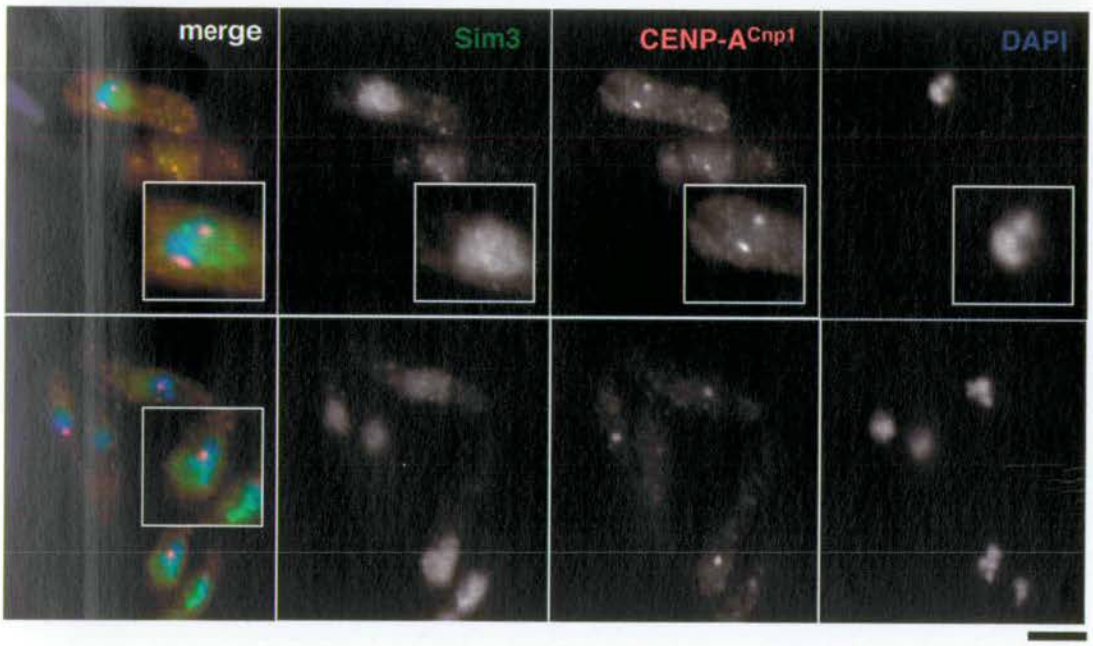


Figure 4-13. Sim3 does not appear to be enriched at centromeres.

A. Wild type cells were fixed and co-stained with anti-Sim3 (green) and anti-CENP-A^{Cnp1} (red) and DNA was stained with DAPI (blue). Co-localisation between the uneven nuclear staining pattern of Sim3 and CENP-A^{Cnp1} spots were never observed.

B. *nda3-311* cold sensitive mutants were grown overnight at 18°C, fixed and co-stained with anti-Sim3 (green) and anti-CENP-A^{Cnp1} (red) and DNA was stained with DAPI (blue). *nda3-311* mutants allow the three fission yeast chromosomes to be visualised. Sim3 was not localised specifically with the chromatin and co-localisation between Sim3 nuclear spots and CENP-A^{Cnp1} spots were never observed. Bar, 5 μm.

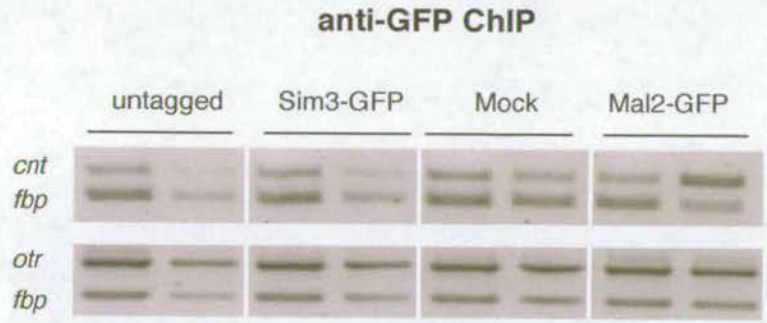


Figure 4-14. Sim3-GFP is not enriched at central core or outer repeats sequences of the centromere by chromatin immunoprecipitation.

Anti-GFP ChIP was carried out on *sim3*$^{+}$-GFP strain to determine whether Sim3 is specifically associated with centromeric sequences. Immunoprecipitated DNA was analysed by multiplex PCR with primer pairs specific for the central core (*cnt*) or outer repeat (*otr*) sequences and *fbp1* (*fbp*) was used as a euchromatic negative control locus. No enrichment for Sim3-GFP was detected at *cnt* or *otr* regions. For mock sample, lysate from Sim3-GFP was incubated with beads only (no antibody). The kinetochore protein Mal2-GFP was enriched at *cnt* but not *otr* and was used as positive control.

centromere, Sim3 was over-expressed in wild type, *sim3-143*, *sim3-205* and a selection of kinetochore mutants known to alleviate central core silencing (*sim2*, *sim4* and *mis6*). *sim2-76* and *sim2-87* have point mutations in the histone fold domain of CENP-A^{Cnp1}, which has been shown to be required for CENP-A targeting to centromeres in HeLa cells (Sullivan et al., 1994). *sim2-169* carries a point mutation in helix 1 of CENP-A^{Cnp1} (A. Pidoux, unpublished result). The *sim3*$^+$ ORF was cloned into the pREP41X *nmt1*$^+$ promoter plasmid, which induces a medium level of protein expression and plasmid was then sequenced to ensure no mutations had occurred. Levels of Sim3 over-expression from pREP41X plasmid are shown by anti-Sim3 western blot analysis (cells were cultured in media lacking thiamine to induced expression), (Figure 4-15). High levels of Sim3 over-expression were found to be toxic in wild type and in kinetochore mutants analysed. *sim3* mutants were only partially complemented by a high level of Sim3 over-expression, which is not surprising as toxic effects were observed even in wild type cells (Figure 4-15).

In order to determine effect of lower level of over-expression of Sim3, a multicopy genomic plasmid (rescued from Shimoda library) was transformed into in wild type cells and in kinetochore mutants known to alleviate silencing. Levels of Sim3 over-expression from the genomic plasmid are shown by anti-Sim3 western blot analysis (Figure 4-16). Low levels of Sim3 over-expression had no adverse effect on growth of the wild type but resulted in decreased viability on Phloxin B of *sim2* and *sim4* strains but not *mis6-302* (Figure 4-16).

4.13 *sim3* mutants are sensitive to over-expression of histone H3, but not H4, and are rescued by CENP-A^{Cnp1} over-expression

As Sim3 shows homology to histone chaperone, it was of interest to determine if *sim3* mutants were sensitive to or could be rescued by the supply of an excess of histones in the cell. Histones H3, H4, H2A, H2B and CENP-A^{Cnp1} were expressed from the *nmt1*$^+$ promoter in pREP41X and pREP3X *LEU2*$^+$ plasmids (cloned and sequenced by Alison Pidoux). *nmt1*$^+$ promoter expression is repressed in media containing thiamine but induced in media lacking thiamine. The level of expression from pREP41X is approximately 10X less than that of pREP3X due to mutations in TATA box of *nmt1*$^+$ promoter (Basi et al., 1993). The plasmids (along with control empty vector plasmids pREP41X or pREP3X) were transformed into *sim3* mutants and transformants were selected on medium lacking leucine supplemented with thiamine (15 μM). Colonies were then plated by serial dilution on medium lacking leucine and lacking thiamine, to derepress protein expression. The effect of histone over-expression on cell viability and silencing was then assessed.

4.13.1 pREP41X-histone (medium level) of histone over-expression in *sim3* mutants

Previously, over-expression of Sim2/CENP-A^{Cnp1} was shown to fully suppress the

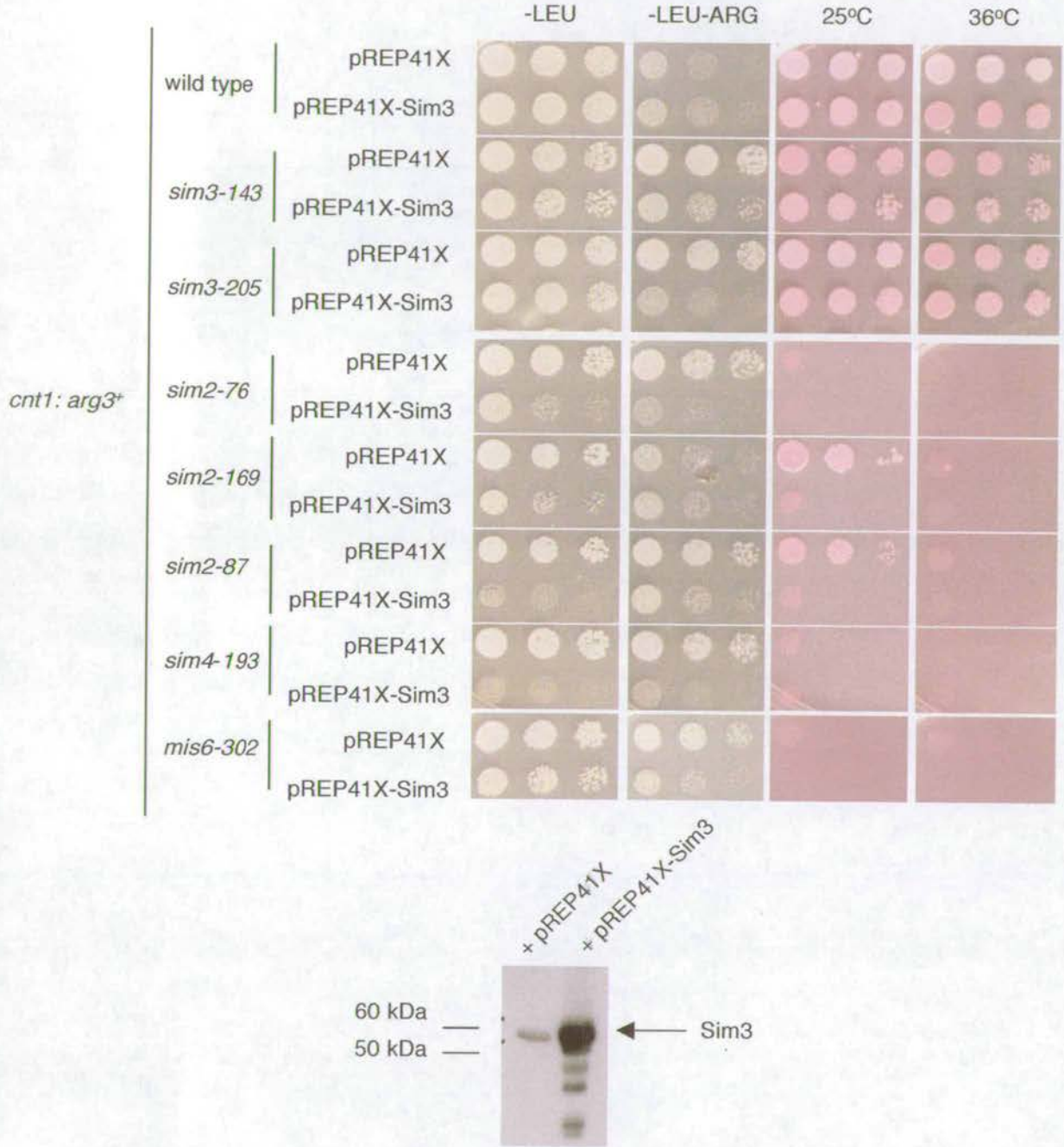


Figure 4-15. Analysis of medium level over-expression of Sim3 in wild type and kinetochore mutants.

Wild type (3027), *sim3*, *sim2*, *sim4* and *mis6* strains were transformed with pREP41X-Sim3 plasmid (*LEU2*+) and colonies were spotted onto medium lacking arginine (no thiamine) at 25°C, to assay central core silencing, and growth on Phloxin B (PMG-LEU, no thiamine) at high temperatures to assay ts. Strains were also transformed with control empty *LEU2*+ plasmid (pREP41X). Level of Sim3 over-expression was assessed by anti-Sim3 western blot. 5 x 10^6 cells were loaded per lane.

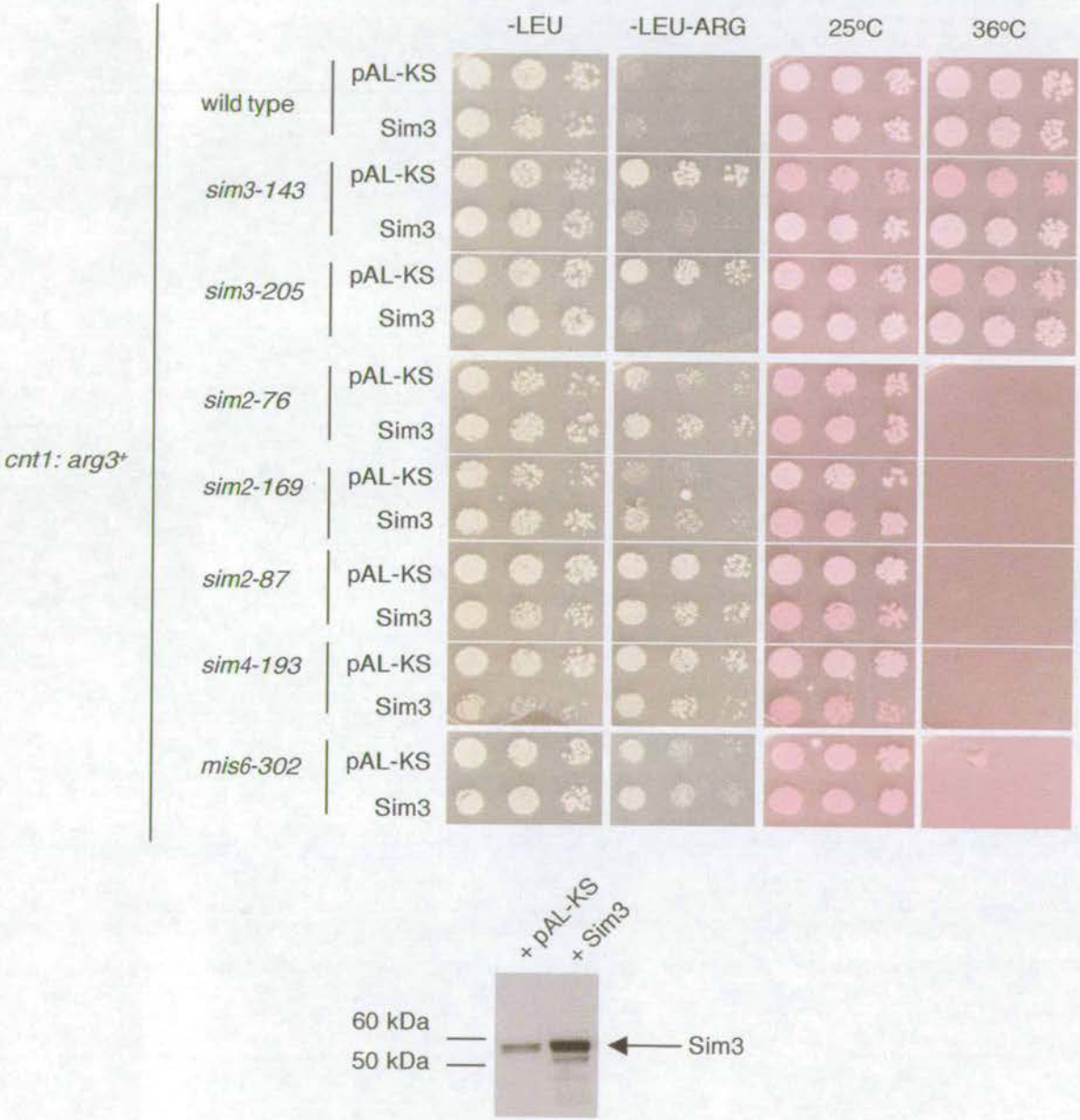


Figure 4-16. Analysis of low level over-expression of Sim3 in wild type and kinetochore mutants.

Wild type (3027), *sim3*, *sim2*, *sim4* and *mis6* strains were transformed with high copy *LEU2*+ genomic plasmid (called here Sim3) and colonies were spotted onto medium lacking arginine at 25°C, to assay central core silencing, and growth on Phloxin B (PMG-LEU) at high temperatures to assay ts. Strains were also transformed with control empty *LEU2*+ plasmid (pAL-KS). Level of Sim3 over-expression was assessed by anti-Sim3 western blot. 5 x 10^6 cells were loaded per lane.

temperature sensitive phenotype of *sim2-76*, but only partially suppress the temperature sensitive phenotype of *sim3-143*, *sim4-193* and *mis6-302* (Pidoux et al., 2003). Here, over-expression of CENP-A^{Cnp1} was found to re-impose silencing at the central core domain in both *sim3* mutants and resulted in only slightly better growth at 32°C, compared to control empty plasmid. Over-expression of histone H3 resulted in poorer growth of *sim3* mutants, which appeared a darker pink on phloxin at 32°C. In contrast, over-expression of histone H4 resulted in slightly better growth on phloxin at 32°C. Alteration of H3 and H4 levels showed a subtle reduction in the alleviation of silencing at the central core, as monitored by slower growth on media lacking arginine. Low levels of over-expression of histone H2A and histone H2B were slightly toxic to the growth of the *sim3-143* mutant but had no effect on the growth of *sim3-205*, suggesting that *sim3* mutations may have allele specific effects (Figure 4-17).

4.13.2 pREP3X-histone (high level) of histone over-expression in *sim3* mutants

A high level of CENP-A^{Cnp1} over-expression was found to be toxic to the growth of both wild type and *sim3* mutants at 32°C. A high level of H3 over-expression was also extremely toxic to the growth of *sim3* mutants, even at the permissive temperature of 25°C. In contrast, over-expression of histone H4 slightly improved the growth of *sim3* mutants at higher temperatures but had no effect on silencing. Interestingly, as observed with medium levels of over-expression, high levels of histone H2A and histone H2B were toxic to the growth of the *sim3-143* mutant but exerted no adverse effects on the growth of *sim3-205*, suggesting that *sim3* mutants may display allele specific effects (Figure 4-18).

DISCUSSION

sim3 mutants alleviate central core silencing and have defects in chromosome segregation

This chapter describes the initial characterisation of the *sim3* mutant phenotype and the identification of the $sim3^{+}$ gene. *sim3* mutants were isolated from a screen in which transcriptional silencing was exploited to facilitate the identification of mutants that affect centromere-kinetochore function (Pidoux et al., 2003). The *sim3* mutant was found to specifically alleviate silencing at the central core domain, and have no effect on silencing at heterochromatic silenced loci. In addition, the *sim3* mutant shows elevated rates of chromosome loss, uneven segregation of chromosomes, a high rate of lagging chromosomes on late anaphase spindles and sensitivity to the microtubule destabilizing drug TBZ. In general, mutants that alleviate central core silencing such as *mis6* and *sim2*/*CENP-*A^{cnp1} are not sensitive to MT destabilization and display uneven chromosome segregation with few lagging chromosomes. This suggests that kinetochore-MT interactions are relatively intact

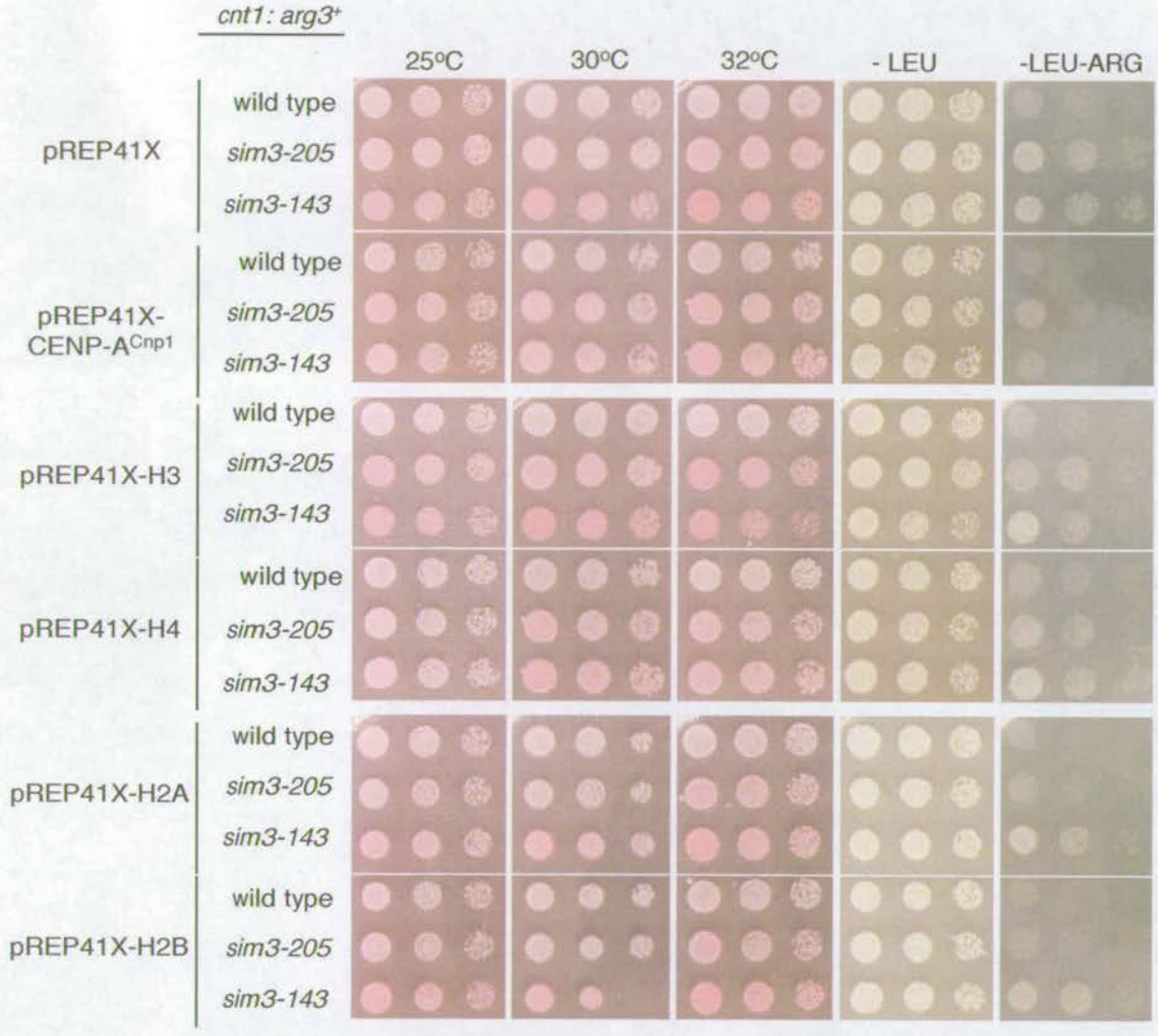


Figure 4-17. Analysis of medium level of histone over-expression in *sim3* mutants (pREP41X).

Strains were transformed with *LEU2*+ pREP41X-histone plasmids (and empty plasmid pREP41X control) and plated on media containing thiamine. Cells were then spotted onto medium lacking thiamine to induce over-expression. Wild type (3027) and *sim3* mutant strains used have an *arg3*+ gene inserted at the central core of chromosome 1 to allow alleviation of silencing at the central core to be monitored by growth on medium lacking arginine (-LEU-ARG, no thiamine) at 25°C. Effects on temperature sensitivity was assayed by growth on -LEU containing Phloxin B (no thiamine) at 25°C, 30°C and 32°C.

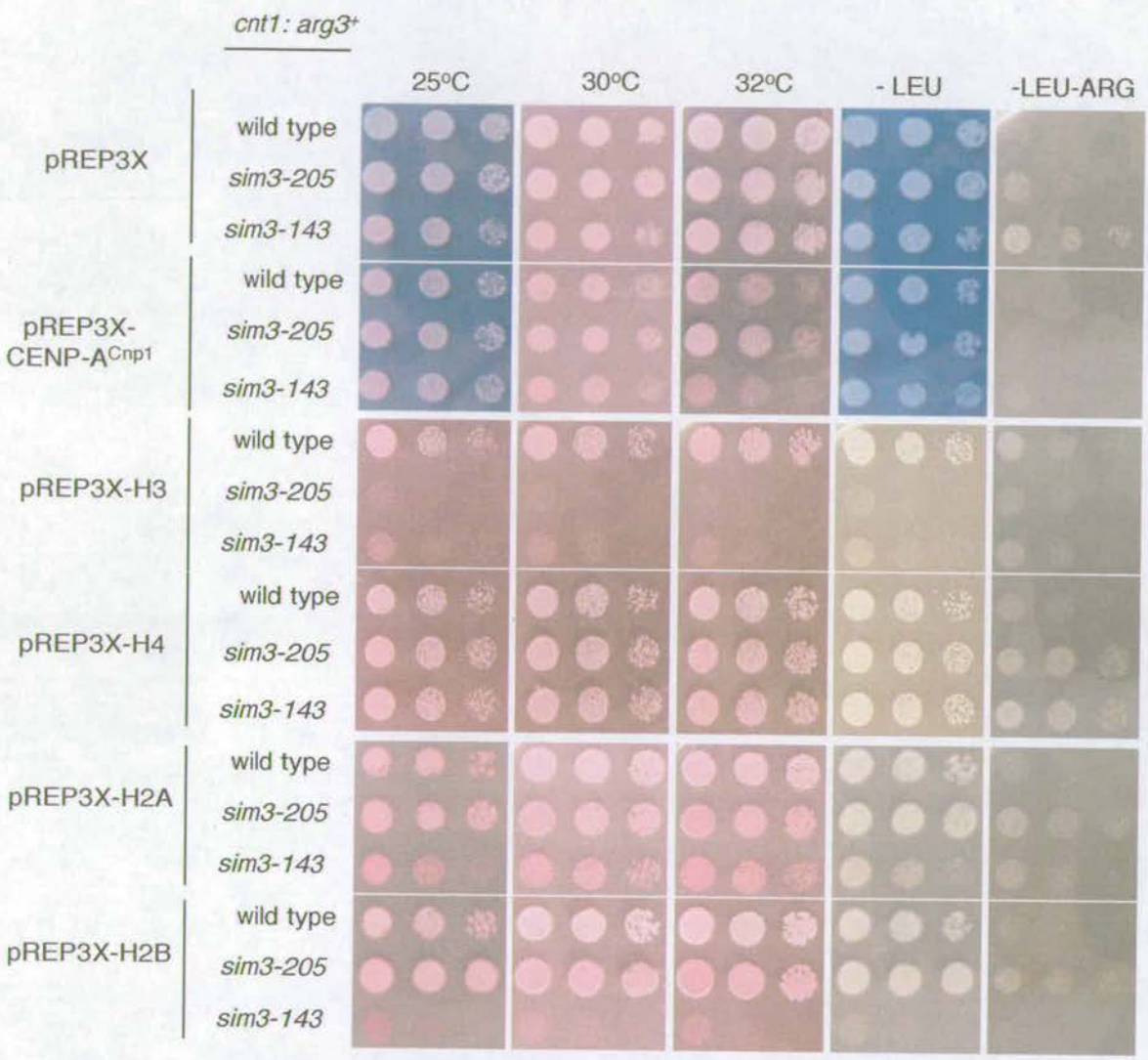


Figure 4-18. Analysis of high level of histone over-expression in *sim3* mutants (pREP3X).

Strains were transformed with *LEU2*+ pREP3X-histone plasmids (and empty plasmid pREP3X control) and cells were spotted onto medium lacking thiamine to induce over-expression. Wild type (3027) and *sim3* mutant strains used have an *arg3*+ gene inserted at the central core of chromosome 1 to allow alleviation of silencing at the central core to be monitored by growth on medium lacking arginine (-LEU-ARG, no thiamine) at 25°C. Effects on temperature sensitivity was assayed by growth on -LEU containing Phloxin B (no thiamine) at 25°C, 30°C and 32°C.

but biorientation is defective in this class of mutants. In contrast, mutants that alleviate outer repeat silencing, such as *rik1Δ*, have high levels of lagging chromosomes and are supersensitive to MT-disrupting drugs, perhaps indicative of defects in MT interaction or in the coordination of MT-binding sites on kinetochores (Allshire et al., 1995, Ekwall et al., 1995, 1996). This may be due to the fact that Swi6 heterochromatin is required to maintain centromeric cohesion, by recruiting Rad21-cohesin (Bernard et al., 2001, Nonaka et al., 2002), which may be required for the maintenance of proper MT attachments. *sim3* mutants fall into a different class as they display both uneven segregation and lagging chromosomes and are sensitive to MT drugs. This suggests that Sim3 may be important for establishing both kinetochore-MT interactions and kinetochore biorientation or may be acting very early in the formation of a centromere in order to affect both of these downstream processes.

Sim3 is the fission yeast homologue of mammalian NASP

The gene encoding *sim3*$^{+}$ was cloned by complementation using a high copy genomic library and the Sim3 was identified as the fission yeast homologue of mammalian nuclear autoantigenic sperm protein (NASP) and *Xenopus laevis* N1/N2, both of which have been shown to have histone chaperone activities. N1/N2 has been shown to facilitate the transfer of histones H3 and H4 to DNA and has been implicated in chromatin assembly *in vitro* (Kleinschmidt et al., 1985; Kleinschmidt et al., 1990). NASP was first described as a cell cycle regulated, linker histone H1 binding protein, which can transfer histone H1 to DNA *in vitro* (Richardson et al., 2000). NASP occurs in two major forms: testicular NASP (tNASP) found in gametes and in the embryo and a shorter version called somatic NASP (sNASP) found in all rapidly dividing somatic cells, and arises from alternative splicing (Richardson et al., 2000). More recently, both tNASP and sNASP were found to co-purify with the histone H3.1 and histone H3.3 complexes, which facilitate replication dependent and independent H3 chromatin assembly respectively (Tagami et al., 2004). Thus, both N1/N2 and NASP have histone chaperone activities and have roles in chromatin assembly. Due to the homology that Sim3 shows to NASP and N1/N2, this suggests that Sim3 may also function as a histone chaperone.

Sim3 was found to localize to the nucleus throughout the cell cycle in both fixed cells stained with anti-Sim3 and in live cells expressing Sim3-GFP. Both NASP and N1/N2 were previously reported to localize to the nucleus and appear to fill the entire nucleus (Richardson et al., 2000, Kleinschmidt et al., 1990). In HeLa cells it has also been shown that both sNASP and tNASP mRNA is cell cycle regulated, increasing during S phase and declining in G2 concomitant with histone mRNA levels (Richardson et al., 2000). Sim3 is localised to the nucleoplasm throughout the cell cycle and does not appear to be cell cycle regulated by immunostaining. Sim3 does not appear to be associated with chromatin as analysis of both fixed and live cells indicated that the Sim3/Sim-GFP signal did not

perfectly colocalise with the DAPI signal but overlapped with it, filling the spherical nuclear volume. In addition, in *nda-3KM311* cold sensitive mutants, Sim3 was not specifically localized to the three highly condensed chromosomes but filled the entire nucleus. Moreover, permeabilised *sim3*$^{+}$-GFP cells treated with 1% Triton-X-100 and viewed by fluorescence microscopy revealed that Sim3-GFP was easily washed out of the nucleus and did not remain chromatin bound (A. Pidoux unpublished result). Kinetochore proteins such as Mis6 and Sim4 are localized to the kinetochore and *mis6* and *sim4* mutants have been shown to alleviate silencing at the central core (Partridge et al., 2000, Pidoux et al., 2003). As *sim3* mutants specifically alleviate central core silencing it is possible that Sim3 may localise to the centromere, however no co-localisation between Sim3 and anti-CENP-A^{Cnp1} was detected cytologically and Sim3-GFP was not enriched at the centromere by chromatin IP. As alleviation of central core silencing is often indicative of a defect in CENP-A ^{Cnp1} association with the central domain, the most obvious explanation for the *sim3* silencing defect is due to a failure to properly localize CENP-A^{Cnp1} (addressed in chapter 5).

In *sim3* mutants, particularly at the restrictive temperature, an increased amount Sim3 protein was detected in the cytoplasm by immuno-fluorescence microscopy. This increase in cytoplasmic signal was also observed by live imaging of *sim3-143*^{ts}-GFP cells at 32°C but not at the permissive temperature of 25°C. This suggests that at higher temperatures, although still produced, the mutant Sim3 protein is less efficient at getting to the nucleus or may get to the nucleus but can no longer be efficiently retained there. Another possibility is, that mutant Sim3 protein is mis-folded and accumulates in the cytoplasm where it is targeted for degradation. The decrease in the amount of functional Sim3 protein in the nucleus may contribute to the downstream effect of this mutant on chromatin integrity.

Sim3 may function at the centromere/kinetochore

Previously, genetic interactions between Sim3 proteins and other kinetochore proteins, indicated that Sim3 was likely to function at the kinetochore or play a role in kinetochore integrity (Pidoux et al., 2003). As a result, the ability of Sim3 to complement the phenotypes of known kinetochore mutants was analysed by Sim3 over-expression. The initial observation that high levels of Sim3 over-expression were toxic to the growth of wild type cells suggested that maintaining the correct level of Sim3 or proteins it regulates is important for the viability of the cell. Lower levels of Sim3 over-expression, which had no adverse effects on wild type growth, resulted in poorer growth of *sim2/CENP-A*^{cnp1} and *sim4* mutants. The toxic effect that results from the over-expression of a candidate gene in a target mutant strain is known as synthetic dosage lethality (SDL) (Kroll et al., 1996). A SDL interaction is often indicative that two genes are acting in the same pathway or both are interacting with a common protein. It seems logical that as Sim3 contributes to the proper integrity of chromatin at centromeres, the kinetochore components such as CENP-A^{Cnp1} and

Sim4, which assemble at a functional centromere are also sensitive to Sim3 protein levels. The results of the analyses of over-expression of Sim3 imply a role for Sim3 at the centromere-kinetochore. It is also possible that Sim3 is in a complex with CENP-A^{Cnp1} or Sim4. If so, the over-expression of Sim3 may titrate the amount of CENP-A^{Cnp1} or Sim4 available for targeting to the kinetochore as levels of these kinetochore proteins may be limiting in the cell.

Sim3 may function as a histone chaperone

Sim3 shows homology to conserved histone chaperones NASP and N1/N2. If Sim3 is also acting as a chaperone, it may function by folding histones correctly, by protecting histones from becoming denatured or by delivering them to chromatin. As a result, increasing the levels of specific histones in the cell may compensate for these impaired functions in *sim3* mutants. Thus, the effect of altering levels of histones on the alleviation silencing and growth of *sim3* mutants was assessed (Figure 4-19).

Over-expression of CENP-A^{Cnp1} (pREP41X) resulted in the restoration of silencing at the central core in *sim3* mutants, suggesting that in *sim3* mutants the levels of CENP-A^{Cnp1} at the centromere may be reduced. The supply of extra H4 to *sim3* mutants resulted in a subtle improvement in growth at 32°C. This is consistent with the finding that supply of histone H4 was found to complement the growth defects of a *cnp1-1* temperature sensitive mutant (Takahashi et al., 2000). Whether this particular CENP-A^{Cnp1} point mutation gives rise to alleviation of silencing has not yet been assessed, but these mutants do display disrupted centromere chromatin, as assayed by MNase digestion patterns (Takahashi et al., 2000). It is known however, that other point mutations in *CENP-A^{cnp1}* alleviate silencing, thus we can infer that the supply of H4 can rescue cells with defective CENP-A^{Cnp1}.

The supply of extra H3 was found to be extremely toxic to *sim3* mutants, as it is in *sim2/CENP-A^{cnp1}* mutants (A. Pidoux, unpublished observation). A similar effect has been reported in budding yeast, where over-expression of H4 improves the viability of a *cse4* mutant strain and over-expression of H3 is toxic to the growth of a *cse4* mutant strain (Glowczewski et al., 2000, Meluh et al., 1998, Smith et al., 1996). This toxicity may result from the fact that the overproduction of H3 is titrating the amount of H4 available to bind to CENP-A^{Cnp1} (a competitor for H4 binding), reducing the amount of CENP-A^{Cnp1} available for targeting to the centromere and resulting in loss of cell viability. Consistent with this, the supply of H4 (a binding partner of CENP-A^{Cnp1}) did not exert a similar toxic effect. In fact, over-expression of H4 was found to complement the *cnp1-1* temperature sensitive mutant (Chen et al., 2003), presumably due to the increased concentration of H4 available for binding to CENP-A^{Cnp1}, which promotes CENP-A^{Cnp1} assembly. Thus, the analyses of histone over-expression in *sim3* mutants are consistent with the role for Sim3 acting as a

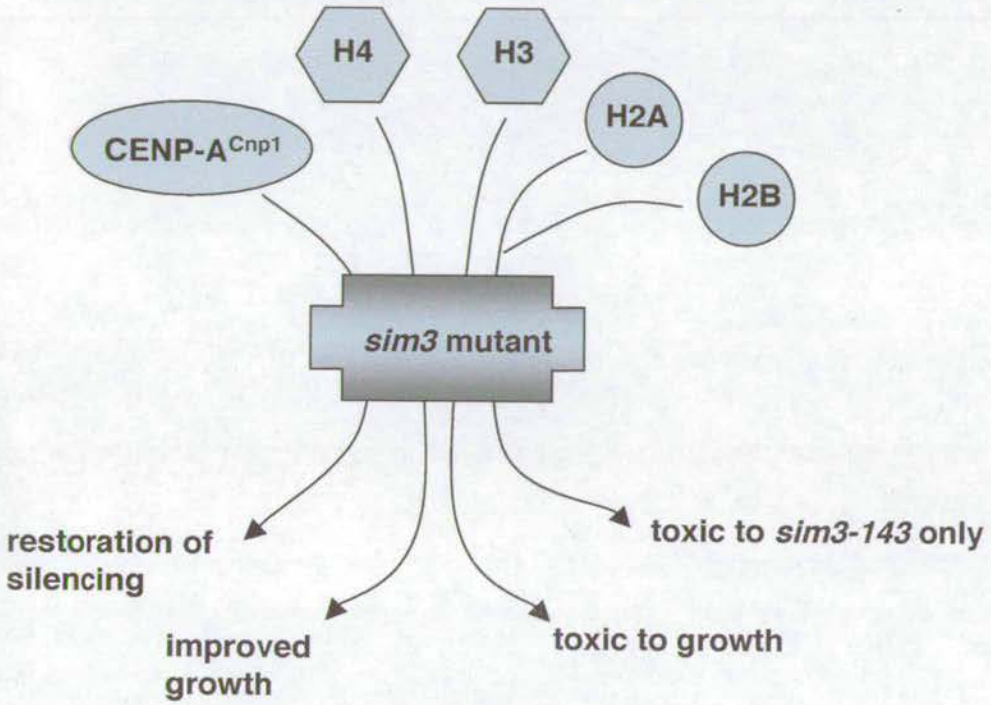


Figure 4-19. Histone over-expression in *sim3* mutants.

Over-expression of CENP-A^{Cnp1} in *sim3* mutants results in the restoration of silencing at the central core region but does not complement temperature sensitivity of the mutants. Over-expression of histone H4 in *sim3* mutants results in a subtle improvement in growth at higher temperatures. Over-expression of H3 is toxic to the growth of *sim3* mutants. Over-expression of H2A and H2B is toxic to the growth of *sim3-143* only and does not adversely affect the growth of *sim3-205*. See text for a more detailed discussion of phenotypes.

CENP-A^{Cnp1} delivery partner. The ability of Sim3 to act as a histone chaperone, and more specifically, as a CENP-A^{Cnp1} chaperone due to its effects of centromere silencing, is investigated and discussed in chapter 5.

The observation that increased levels of histone H2A and histone H2B were toxic to the growth of the *sim3-143* mutant, but exerted no adverse effects on the growth of *sim3-205* was striking and suggests that different *sim3* temperature sensitive point mutations may disrupt specific functions. Interestingly *sim2/CENP-A^{cnp1}* mutants are also sensitive to H2B over-expression (A. Pidoux, unpublished observation) and mutations in H2B itself have recently been shown to alleviate silencing at the central core domain (Maruyama et al., 2006). The *sim3-143* point mutation results in non-functional and possibly incorrectly folded Sim3 protein at the restrictive temperature that may differ from the mutant protein resulting from the *sim3-205* point mutation. The expression of extra H2A and H2B may further compromise Sim3 function in *sim3-143* at the permissive temperature, possibly by directly binding to and sequestering Sim3 or by an indirect interaction with a common protein.

CHAPTER 5

ROLE OF Sim3 IN CENP-A^{Cnp1} CHROMATIN ASSEMBLY

INTRODUCTION

CENP-A is the histone H3 variant that specifically replaces H3 in centromeric nucleosomes (Palmer et al., 1991, Sullivan et al., 1994). The specialized CENP-A chromatin forms the platform upon which the kinetochore is assembled. However it is unknown how exactly CENP-A is targeted to and assembled only at the centromere and excluded from non-centromeric chromatin. It was previously proposed that CENP-A could be targeted to centromeres by coupling the peak of its expression to the late replication of centromeres, which would favour the preferential incorporation of CENP-A (Shelby et al., 1997). However, it was later shown that centromeric DNA replication is uncoupled from CENP-A synthesis (Shelby et al., 1997, Shelby et al., 2000) and that both human and fly CENP-A can be efficiently loaded in G2 in the absence of DNA replication (Shelby et al., 2000, Ahmad and Henikoff, 2001). Subsequently, the incorporation of CENP-A in G2 has been demonstrated in fission yeast and *Arabidopsis thaliana* (Takahashi et al., 2005, Lermontova et al., 2006). Analyses in mammalian cells have also shown the histone fold domain of CENP-A to be essential for targeting of CENP-A (Sullivan et al., 1994, Black et al., 2004). Recently, the CENP-A nucleosome associated complex has been identified in HeLa cells (Foltz et al., 2006). Members of the complex were shown to localize to the centromere and are required for kinetochore integrity (Foltz et al., 2006), however the role of these proteins in CENP-A chromatin assembly has not been investigated. In fission yeast, a number of factors have been described that contribute to CENP-A incorporation at centromeres, including the Mis6-Sim4 complex (Takahashi et al., 2000, Pidoux et al., 2003), the Mis16-Mis18 complex (Hayashi et al., 2004) and the Ams2 GATA-like factor (Chen et al., 2003, Takahashi et al., 2005).

The kinetochore protein Mis6 has been shown to be required for the localization of newly produced CENP-A^{Cnp1} to centromeres (Takahashi et al., 2000) and is found in a complex with Sim4 (Pidoux et al., 2003, Liu et al., 2005) and Mis15 and Mis17 (Hayashi et al., 2004). Depletion of any component of the Mis6 complex leads to a reduction in the amount of CENP-A^{Cnp1} at centromeres (Takahashi et al., 2000, Pidoux et al., 2003, Hayashi et al., 2004). It was previously reported that chicken CENP-I, the Mis6 counterpart in human cells, was not required for the localization of CENP-A (Nishihashi et al., 2002) and was proposed to function downstream of CENP-A in a complex with the kinetochore protein CENP-H (Regnier et al., 2005). However, the recent purification of the CENP-I-CENP-H complex from human and chicken cells has identified 11 interacting proteins that constitutively

localize to centromeres, including CENP-K, the Sim4 counterpart in human cells (Okada et al., 2006). This recent data now indicates that components of the CENP-I-H complex (CENP-H, CENP-I, CENP-K and CENP-M) play a role in the efficient incorporation of newly synthesized CENP-A at centromeres in chicken DT40 cells, despite the earlier observation that there was no reduction in the levels of endogenous CENP-A at kinetochores (Okada et al., 2006). This provides evidence that Mis6/Sim4-containing complex may have a conserved role in ensuring CENP-A incorporation at centromeres.

Mutations in *S. pombe mis16* and *mis18* also result in the delocalization of CENP-A^{Cnp1} from centromeres (Hayashi et al., 2004). *mis16*$^{+}$ encodes a homologue of human retinoblastoma binding proteins RbAp46/RbAp48 (Qian et al., 1993, Qian and Lee, 1995) and kinetochore localization of CENP-A is abolished upon double knockdown of RbAp46/RpAb48 in HeLa cells (Hayashi et al., 2004). RbAp46/RbAp48 have been reported as members of various chromatin-modifying complexes that have histone deacetylase, histone acetylase and nucleosome positioning activities (Loyola and Almouzni, 2004). In addition, RbAp48 is a component of the human chromatin assembly factor 1 (CAF-1) complex which functions in replication coupled assembly and is also found complexed to HIRA to facilitate transcription-coupled assembly (Tagami et al., 2004). More recently, RbAp48 was purified in a complex with *Drosophila* CENP-A^{CID} and histone H4 and this complex was shown to be sufficient for the assembly of centromeric nucleosomes *in vitro* (Furuyama et al., 2006).

It has been suggested that the pattern of histone modifications at the centromere also contribute to efficient CENP-A chromatin assembly (reviewed Dunleavy et al., 2005). Observations in human, flies and fission yeast has demonstrated that H3 found at the centromere is always in the H3K4 dimethylated state (Blower and Karpen, 2004, Cam et al., 2005) and is proposed to contribute to the three dimensional organization of a functional centromere. In addition, fission yeast Mis16 and Mis18 have been found to be required to maintain the histones H3 and H4 at the central core in a hypoacetylated state (Hayashi et al., 2004). Deletion of fission yeast Hrp1, which shows homology to the CHD (chromo-helicase/ATPase DNA binding) remodelling factor, also results in increased levels of histone H3 and H4 acetylation and reduced CENP-A^{Cnp1} at the central core region of the centromere (Waldfridsson et al., 2005). It is possible that histone deacetylation at the centromere may be a prerequisite for the association of CENP-A^{Cnp1} with centromeres, however histones at the centromere may become hypoacetylated as a consequence of CENP-A^{Cnp1} association.

The cell cycle regulated GATA-type transcription factor Ams2, is another factor implicated in CENP-A^{Cnp1} loading in fission yeast (Chen et al., 2003). *ams2*Δ cells have been shown to be defective in the replication coupled loading on CENP-A^{Cnp1} in S phase (Takahashi et al., 2005). The viability of *ams2*$^{+}$ deleted strains is accounted for by a back-up CENP-A^{Cnp1}-

loading pathway in G2 involving Mis6 that keeps *ams2Δ* cells alive (Takahashi et al., 2005). Thus, fission yeast CENP-A^{Cnp1} can be loaded at the centromere independent of replication, as described in humans and flies (Shelby et al., 2000, Ahmad and Henikoff, 2001). In addition, Ams2 is required for the transcriptional activation of the histone genes during S phase and the cell cycle dependent accumulation of histone mRNAs is diminished in *ams2Δ* cells (Takahashi et al., 2005). The loss of the transcriptional oscillation of the histone genes presumably results in the lack of free histones available for *de novo* chromatin assembly and this alone could account for the reduction in efficiency of CENP-A^{Cnp1} incorporation in *ams2Δ* cells in S phase. Thus, Ams2 is thought to contribute to the loading of CENP-A^{Cnp1} in S phase in a replication dependent manner, at least in part by promoting histone transcription. Also, Ams2 is enriched at centromeres (Chen et al., 2003), so it is possible that it somehow plays a more direct role in the remodelling/assembly of CENP-A^{Cnp1} chromatin.

In summary, the Mis6-Sim4 complex, the Mis16-Mis18 complex, Ams2 and Hrp1 affect the localization of CENP-A^{Cnp1} to fission yeast centromeres, however no direct interaction between CENP-A^{Cnp1} and any of these proteins has been described. Also, the version of CENP-A^{Cnp1} that was used for most analyses is tagged and only partially functional. It is clear that the identity of a factor that both binds to and is required for the assembly of CENP-A at centromeres remains to be determined in both fission yeast and higher eukaryotes. The role of the newly identified CENP-A nucleosome associated proteins from HeLa cells also remain to be uncovered, however this purification was unlikely to identify factors that associate with CENP-A in the pre-deposited form (Foltz et al., 2006). Interestingly, the chromatin remodelling factor FACT (Facilitates Chromatin Transcription) and the nuclear chaperone nucleophosmin-1 were also isolated as part of the complex and may play a direct role in assembly of CENP-A at centromeres (Foltz et al., 2006).

In chapter 4, the phenotype of the *sim3* mutants was described. As previously discussed, Sim3 shows homology to the histone chaperones NASP and N1/N2 and this prompted an investigation into the ability of Sim3 to act as a histone chaperone, in particular a CENP-A^{Cnp1} chaperone. *sim3* mutants specifically alleviate central core silencing and are defective in centromere function, displaying defects in chromosome segregation. In this chapter, I will present results that show that Sim3 is acting as a CENP-A^{Cnp1} associated factor that assists in its delivery to the centromere and discuss its implications. As it has been demonstrated that CENP-A^{Cnp1} can be deposited by a replication-independent mechanism, the contribution of Sim3 to the replication independent incorporation of CENP-A^{Cnp1} was assessed.

RESULTS

5.1 The centromeric histone H3 variant CENP-A^{Cnp1} is delocalised in *sim3* mutants

As both *sim3-143* and *sim3-205* mutants alleviate silencing at the central core domain where the H3 variant CENP-A^{Cnp1} chromatin is specifically assembled, the localisation of CENP-A^{Cnp1} in *sim3* mutants was determined. Affinity purified anti-CENP-A^{Cnp1} antibodies were used to localise CENP-A^{Cnp1} in wild type and *sim3* mutant cells at 25°C and 36°C. Cells were co-stained with anti-Sad1, a spindle pole body marker (Hagan and Yanagida, 1995), which allowed the position of the clustered centromeres at the nuclear periphery to be determined. At permissive temperature, CENP-A^{Cnp1} localisation to centromeres is reduced in intensity and the localisation of CENP-A^{Cnp1} is completely disrupted at restrictive temperature in both *sim3* mutant alleles (Figure 5-1).

In addition, the association of CENP-A^{Cnp1} with the central core domain (*cnt*) and the inner-most repeats (*imr*) was assessed in *sim3* mutants by anti-CENP-A^{Cnp1} chromatin IP (ChIP). Immunoprecipitated DNA was analysed by multiplex PCR and CENP-A^{Cnp1} enrichment at *cnt* and *imr* was determined, measured relative to the euchromatic control *fbp1* and normalized to the input PCR. Consistent with the delocalization of CENP-A^{Cnp1} that was observed cytologically, ChIP analysis showed a reproducible reduction in the association of CENP-A^{Cnp1} with the central core (*cnt*) region of the central domain at both permissive and restrictive temperatures of approximately 10 fold (Figure 5-2A). Quantification of the enrichment of CENP-A^{Cnp1} at *imr* sequences also revealed a 4 fold reduction in the association of CENP-A^{Cnp1} with the inner-most repeat sequences (Figure 5-2B).

As *sim3* mutants show a reduced association of CENP-A^{Cnp1} with centromeres, the amount of histone H3 associated with centromeres in *sim3* mutants was investigated. Chromatin IP using anti-histone H3 antibody raised against the C terminus of H3, was also performed on wild type and *sim3* mutant strains at permissive and restrictive temperature. In the wild type, very little H3 was associated with *cnt* and H3 was found to be enriched at the euchromatic *fpb1* locus as expected. In contrast, elevated levels of histone H3, which is normally under-represented in the central core region, were found to occupy the central core (*cnt*) in *sim3* mutants (Figure 5-2C). These ChIP analyses indicate that there is reduced CENP-A^{Cnp1} at the central domain in *sim3* mutants and an increase in H3. Thus, a function of Sim3 may be to promote the incorporation of CENP-A^{Cnp1} and prevent the incorporation of or facilitate the removal of H3 from centromeres.

5.2 Levels of myc-CENP-A^{Cnp1} protein are not reduced in *sim3* mutants

The amount of CENP-A^{Cnp1} at centromeres is reduced in *sim3* mutants by ChIP and

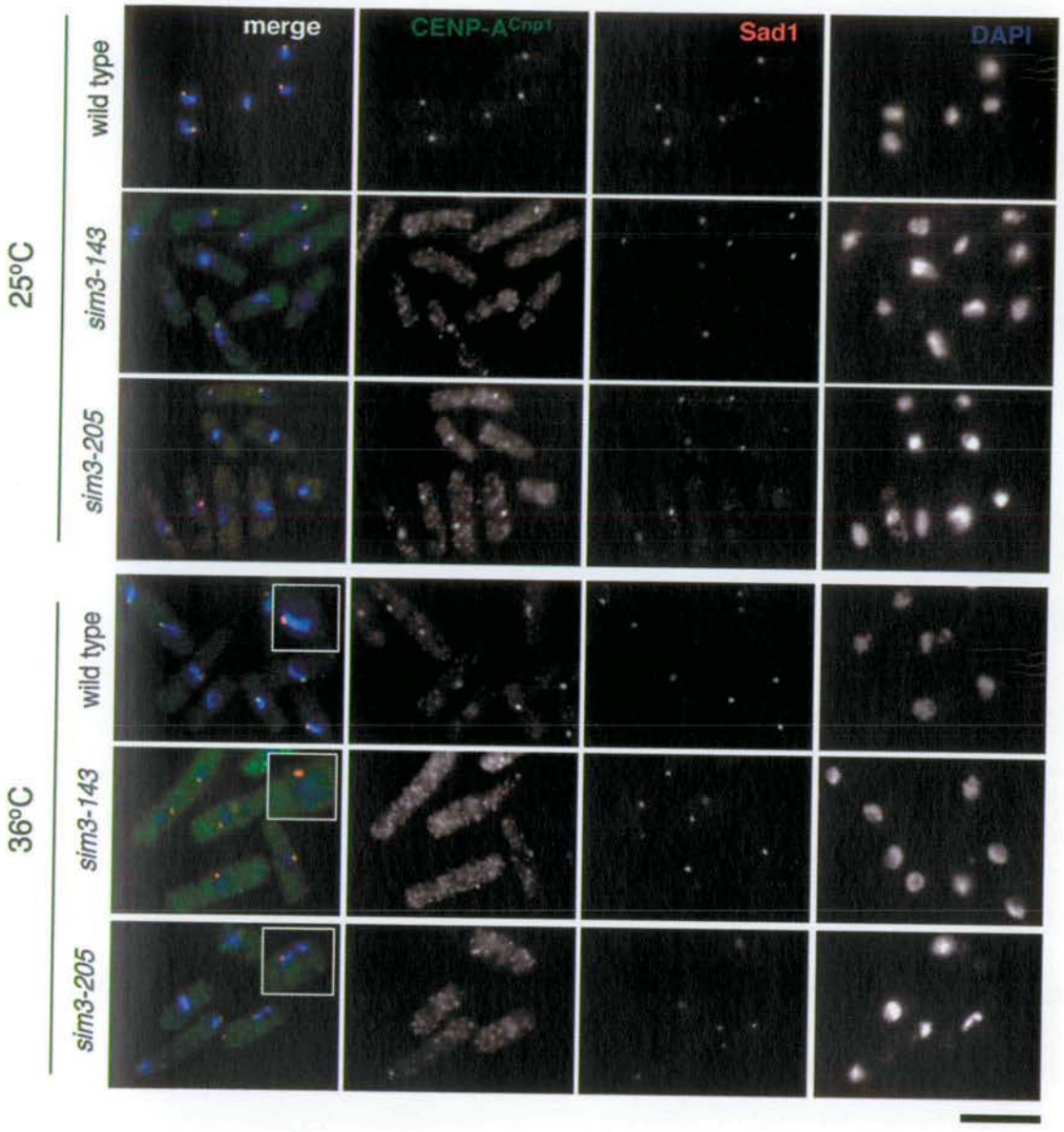


Figure 5-1. Localisation of CENP-A^{Cnp1} in *sim3* mutants at permissive and restrictive temperature.

Cells were grown to log phase at either 25°C or shifted to 36°C for 6 hours, before fixing and staining with affinity purified anti-CENP-A^{Cnp1} (green) and anti-Sad1 (red) and DNA was stained with DAPI (blue). Sad1 staining was used as a marker for centromere position. CENP-A^{Cnp1} signal appears reduced in *sim3* mutants at 25°C and is delocalised at 36°C. Bar 5 μm.

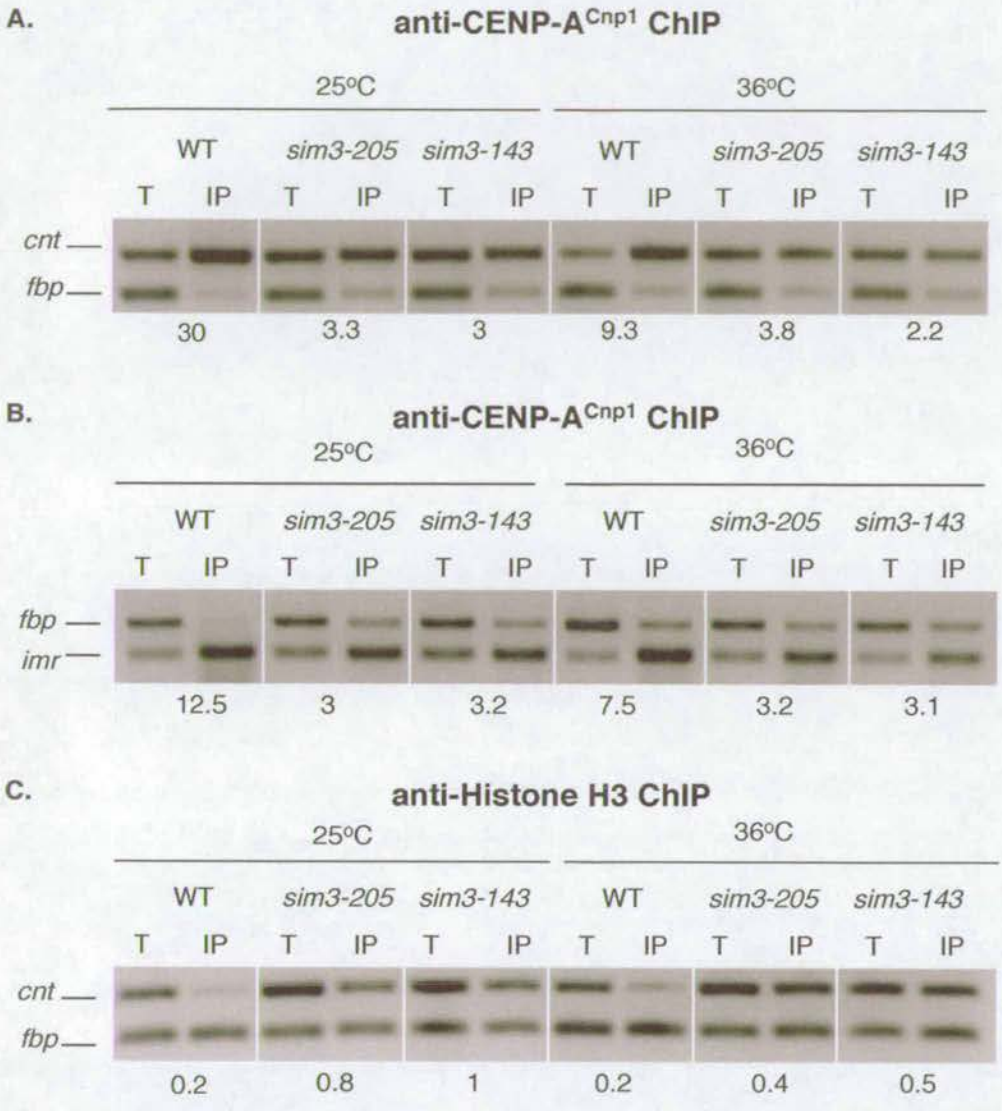


Figure 5-2. Anti-CENP-A^{Cnp1} and anti-H3 chromatin IP performed on *sim3* strains.

Centromere association of CENP-A^{Cnp1} is reduced, and association of histone H3 with the central core domain is increased in *sim3* mutants by chromatin immunoprecipitation at permissive (25°C) and restrictive temperature (36°C for 6 hours). Quantitation of bands was performed using Eastman Kodak Co. EDAS 290 system and 1D Image Analysis software. T = Total input, IP = immunoprecipitate.

A. Chromatin IP was performed with anti-CENP-A^{Cnp1}antibody and precipitated DNA was analysed by PCR with primers for the central core sequences (cntA + cntB) and a euchromatic gene *fpb1*.

B. Chromatin IP was performed with anti-CENP-A^{Cnp1}antibody and precipitated DNA was analysed by PCR with primers for the inner-most repeats at the central core (imrA + imrB) and a euchromatic gene *fpb1*.

C. Chromatin IP was performed with anti-H3 C terminal antibody (AbCam) and precipitated DNA was analysed by PCR with primers for the central core sequences (cntA + cntB) and a euchromatic gene *fpb1*.

immuno-fluorescent microscopy. An alternative and simple explanation for this may be that levels of CENP-A^{Cnp1} protein are reduced in *sim3* mutants and thus there is less CENP-A^{Cnp1} available for targeting to the centromere. The sheep anti-CENP-A^{Cnp1} antibodies utilized in Figure 5-1 was unable to detect endogenous levels of CENP-A^{Cnp1} by western analysis. To circumvent this problem however, two approaches were used. Firstly, a strain with myc-tagged CENP-A^{Cnp1} expressed from its endogenous promoter was used to assess the levels of myc-tagged CENP-A^{Cnp1} protein in *sim3* mutants. Levels of myc-tagged CENP-A^{Cnp1} protein were found to be unaffected by *sim3* mutations at 25°C and 36°C (Figure 5-3A). In addition, levels of CENP-A^{Cnp1} over-expressed from pREP3X were analysed by western blot using affinity purified rabbit anti-CENP-A^{Cnp1} antibody (gift from M. Yanagida). Over-expressed CENP-A^{Cnp1} was detected in wild type and *sim3* mutants at both 25°C and 36°C. Endogenous CENP-A^{Cnp1} was not detected in strains transformed with empty pREP3X plasmid, presumably because CENP-A^{Cnp1}is only present at a very low level in the cell (Figure 5-3B). In addition, levels of histone H3 were assessed in *sim3* mutants by western analysis at permissive and restrictive temperature and were found to be comparable to wild type levels (Figure 5-4).

5.3 Microarray analysis indicates that *sim3* mutants do not affect the expression of known centromere-associated proteins

To test whether *sim3* mutations indirectly affect the levels of CENP-A^{Cnp1} at centromeres via the transcriptional regulation of a gene known to be involved in CENP-A^{Cnp1} chromatin assembly, total RNA was prepared from *sim3-143*, *sim3-205* and an isogenic wild type to enable genome-wide cDNA expression profiling to be performed (in collaboration with Karl Ekwall laboratory). Analysis carried out by the Ekwall laboratory showed that at 36°C, all genes whose expression was 1.5 fold affected by either *sim3-143* or *sim3-205* mutations were not known to affect CENP-A^{Cnp1} chromatin assembly or centromere function (see Table 3). The temperature sensitive kinetochore mutant *mis12-537* that does not affect CENP-A^{Cnp1} levels at centromeres in fission yeast (Goshima et al., 1999) was used as a control for genes that are upregulated in temperature sensitive mutants at the restrictive temperature (data not shown). These results suggest that *sim3* mutants do not indirectly affect the expression of known fission yeast kinetochore proteins or factors that affect CENP-A^{Cnp1} assembly. These results imply that it is most likely that Sim3 is having a direct role in the assembly of central domain chromatin at the centromere. This does not exclude the possibility however, that the Sim3 regulates the expression of an unknown centromere associated factor and does not give any information regarding changes in protein levels in *sim3* mutants.

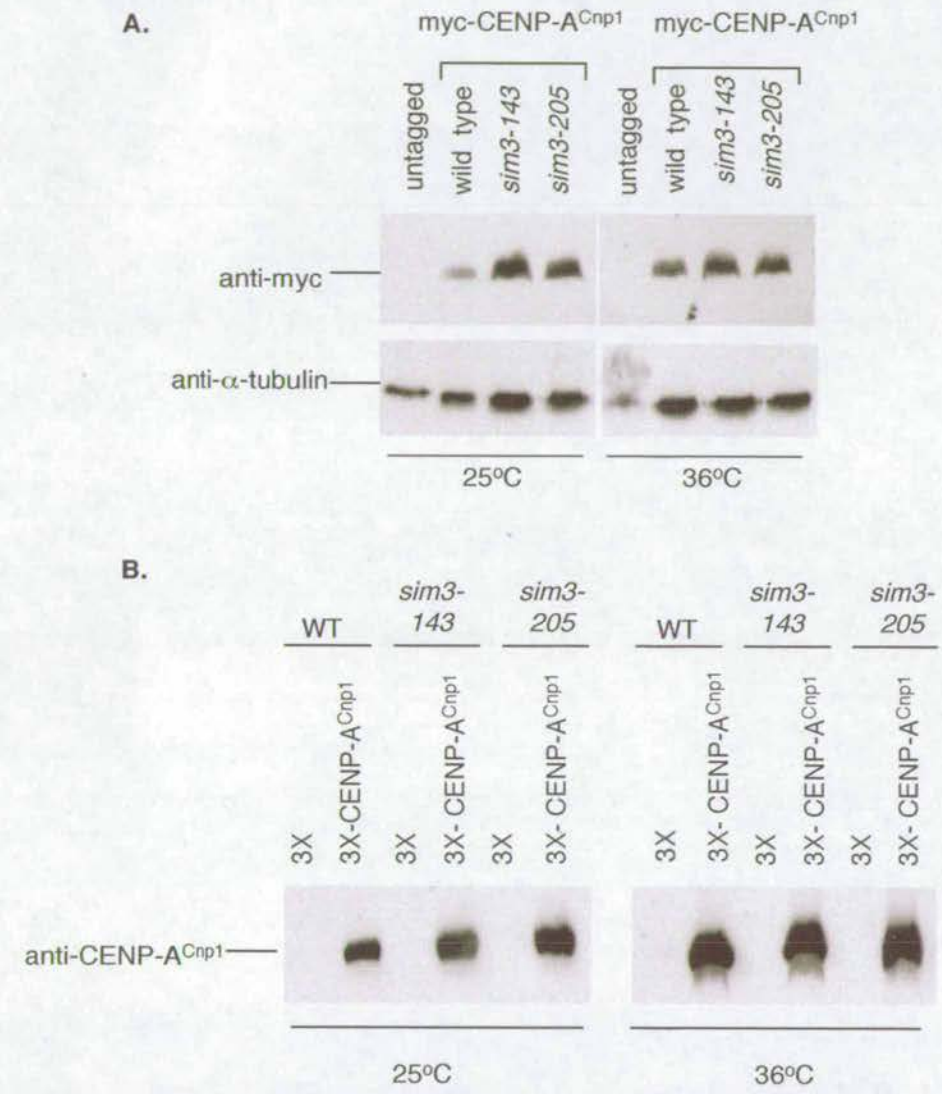


Figure 5-3. Levels of CENP-A^{Cnp1} in *sim3* mutants at permissive (25°C) and restrictive temperature (6 hours at 36°C).

A. Levels of myc-tagged CENP-A^{Cnp1} in *sim3* mutants at permissive and restrictive temperature were tested by anti-myc western blot. CENP-A^{Cnp1} is tagged at its endogenous locus under its own promoter and is the only copy of CENP-A^{Cnp1} in the cell. Both *sim3* mutants alleles show similar levels of myc-tagged CENP-A^{Cnp1} to wild type. Anti-α-tubulin is used as a loading control (anti-TAT1).

B. Levels of CENP-A^{Cnp1} over-expressed from the pREP3X-CENP-A^{Cnp1} inducible plasmid were assessed in *sim3* mutants at permissive and restrictive temperature by affinity purified rabbit anti-CENP-A^{Cnp1} western blot. Levels of over-expressed CENP-A^{Cnp1}were comparable in wild type and mutant at both temperatures. 5 x 10^6 cells were loaded per lane. Endogenous CENP-A^{Cnp1} was not detectable using this antibody.

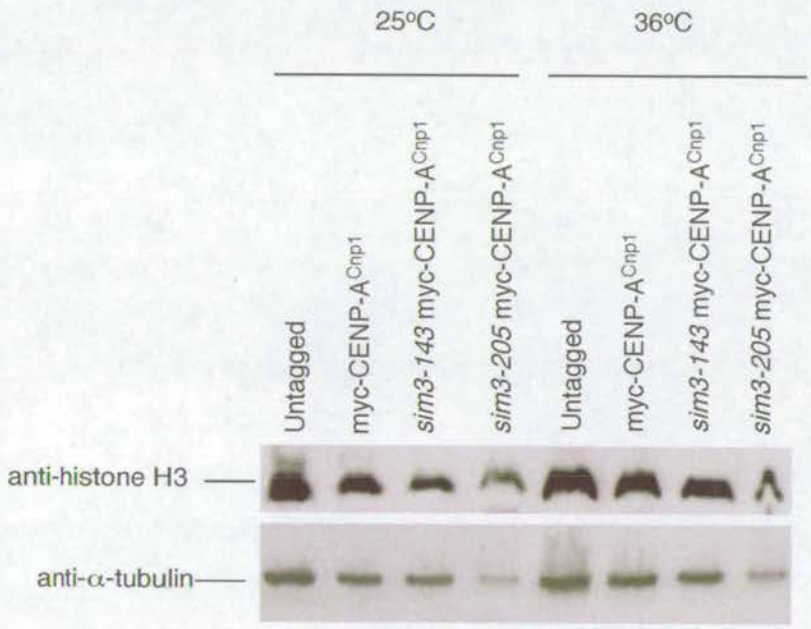


Figure 5-4. Levels of histone H3 in *sim3* mutants at permissive (25°C) and restrictive temperature (6 hours at 36°C).

Levels of histone H3 were assessed in myc-tagged CENP-A^{Cnp1} strain and in *sim3* mutants with myc-tagged CENP-A^{Cnp1} at permissive and restrictive temperature by anti-histone H3 western blot. Both *sim3* mutants alleles show similar levels of histone H3 to wild type. Anti-α-tubulin is used as a loading control (anti-TAT1).

Gene	*sim3-143*	*sim3-205*
CENP-A^{Cnp1}	0.871	0.93
sim4	1.020	1.074
mis6	0.740	1.012
mis16	0.973	0.885
mis18	1.370	0.900
mis12	0.735	1.355
ams2	0.877	0.922
hrp1	1.483	0.72
mis-15	0.998	0.945
mis-17	1.334	1.393
H3 (*hht1*)	0.880	0.974
H3 (*hht2*)	0.944	1.270
H3 (*hht3*)	0.926	1.260
H4 (*hhf1*)	0.980	0.997
H4 (*hhf2*)	0.846	0.826
H4 (*hhf3*)	0.934	0.850
H2A (*hta1*)	0.828	0.733
H2A (*hta2*)	0.842	0.829
H2B (*htb1*)	1.137	0.95

Table 3. *sim3* mutants do not affect the expression of CENP-A^{Cnp1} associated proteins by microarray analysis.

RNA was prepared from *sim3-143* and *sim3-205* mutants grown at the restrictive temperature of 36°C for 6 hours. Two independent RNA preparations were used to hybridise to two microarrays (Eurogentec ORF) one on which wild type was labelled with Cy5 and either *sim3-143* or *sim3-205* with Cy3 and the other on which dyes were swapped. The data were normalized using 'Lowess' (per chip per spot normalizations and checked against luciferase spiked controls). 4 data points were generated for each gene for each *sim3* mutant and the table lists an average of the 4 values. Data points greater than the1.5 cut off value indicate a reproducible change in expression (either high expression or low expression), none of the genes known to be involved in CENP-A^{Cnp1} chromatin assembly in fission yeast were affected. The methodology and arrays were as previously described by Xue et al. (2004).

5.4 Sim3 and CENP-A^{Cnp1} physically interact *in vitro*

Defective Sim3 function leads to loss of CENP-A^{Cnp1} from centromeres and Sim3 shows homology to the histone chaperones NASP and N1/N2. It seemed plausible that Sim3 may act as a chaperone for CENP-A^{Cnp1}. Histone chaperones often carry out delivery functions in the cell and need to bind to their target in order to do so (see review by Loyola and Almouzni, 2004). To determine if Sim3 can bind directly to CENP-A^{Cnp1} or other histones, ^{35}S-labelled Sim3 was produced by *in vitro* transcription and translation and tested for binding to GST-CENP-A^{Cnp1}, GST-H3, GST-H4, GST-H2A and GST-H2B (GST-histone fusion proteins were cloned and sequenced by A. Pidoux). Sim3 was found to bind directly to GST-CENP-A^{Cnp1} and only a slight interaction with GST-H3 was detected (Figure 5-5A). This suggests that Sim3 binds recombinant CENP-A^{Cnp1} more readily than recombinant H3. Sim3 did not bind to GST-H4, GST-H2A or GST-H2B. The binding of mutant ^{35}S-labelled Sim3-143 and Sim3-205 to GST-CENP-A^{Cnp1} was found to be dramatically reduced (Figure 5-5B), which is consistent with a specific interaction between Sim3 and CENP-A^{Cnp1}.

5.5 Sim3 interacts with both CENP-A^{Cnp1} and histone H3 by yeast two hybrid analysis

The interaction between Sim3 and CENP-A^{Cnp1} and Sim3 and histone H3 was also examined by yeast two-hybrid assay. *sim3*$^{+}$, CENP-A^{Cnp1} and histone H3 were cloned into pAS2-1 GAL4 DNA binding domain and pACT2 GAL4 activating domain plasmids and sequenced to ensure that mutations were not present. The *S. cerevisiae* strain PJ69-4A (James et al., 1996) was co-transformed with all possible combinations of plasmids expressing the DNA binding domain and plasmids expressing the activating domain e.g. pAS2-1-Sim3 and pACT2-CENP-A^{Cnp1} and co-transformants were spotted onto selective plates. Growth on media lacking leucine and tryptophan confirmed the presence of the two plasmids. Interaction between pACT2-Sim3 and pAS2-1-CENP-A^{Cnp1} resulted in the expression of the *HIS3* (growth on media lacking leucine, tryptophan and histidine) and *ADE2* (growth on media lacking leucine, tryptophan and adenine) reporter genes. Increasing concentrations of 3-aminotriazol (3-AT), a competitive inhibitor of the imidazole glycerolphosphate dehydratase involved in histidine biosynthesis, were used to inhibit the basal level of expression but not the strong activation of the *HIS3* reporter gene. An interaction between pACT2-Sim3 and pAS2-1-H3 was also detected. The interaction between Sim3 and CENP-A^{Cnp1}, and also, Sim3 and histone H3 was only detected when the histones CENP-A^{Cnp1} and H3 were fused to the DNA binding domain and Sim3 was fused to the activating domain and not in the other orientation (Figure 5-6). Swi6 and Swi2, previously shown to interact by yeast two hybrid assay, were used as positive controls (Jia et al., 2004). These results confirm the *in vitro* interaction between Sim3 and CENP-A^{Cnp1}, but it is unclear if the Sim3-H3 interaction is of physiological significance in *S. pombe*.

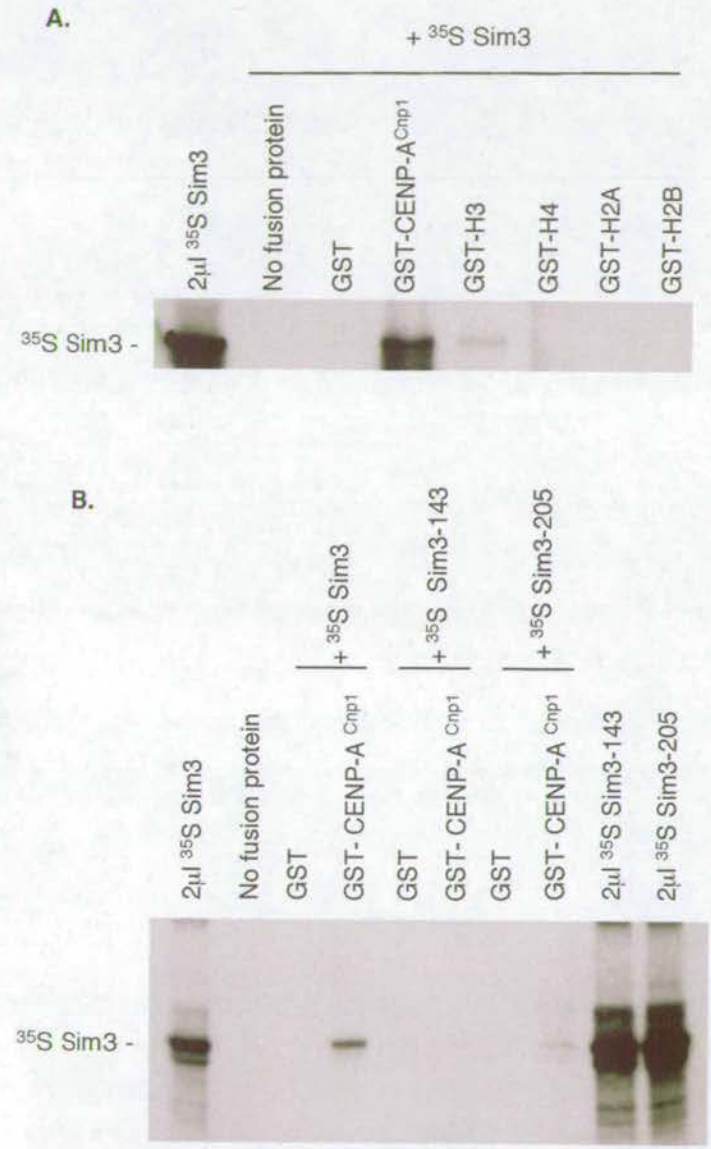


Figure 5-5. Sim3 can interact with CENP-A^{Cnp1} *in vitro*.

A. ^{35}S-labelled Sim3 was produced by *in vitro* transcription and translation and assayed for binding to GST-CENP-A^{Cnp1}, GST-H3,GST-H4, GST-H2A and GST-H2B.

B. Binding of mutant ^{35}S-labelled Sim3-143 and Sim3-205 to GST-CENP-A^{Cnp1} was dramatically reduced in the *in vitro* binding assay. Equal amounts of GST fusion protein (4 µg) and 4 µl of ^{35}S-labelled Sim3 were added to each reaction.

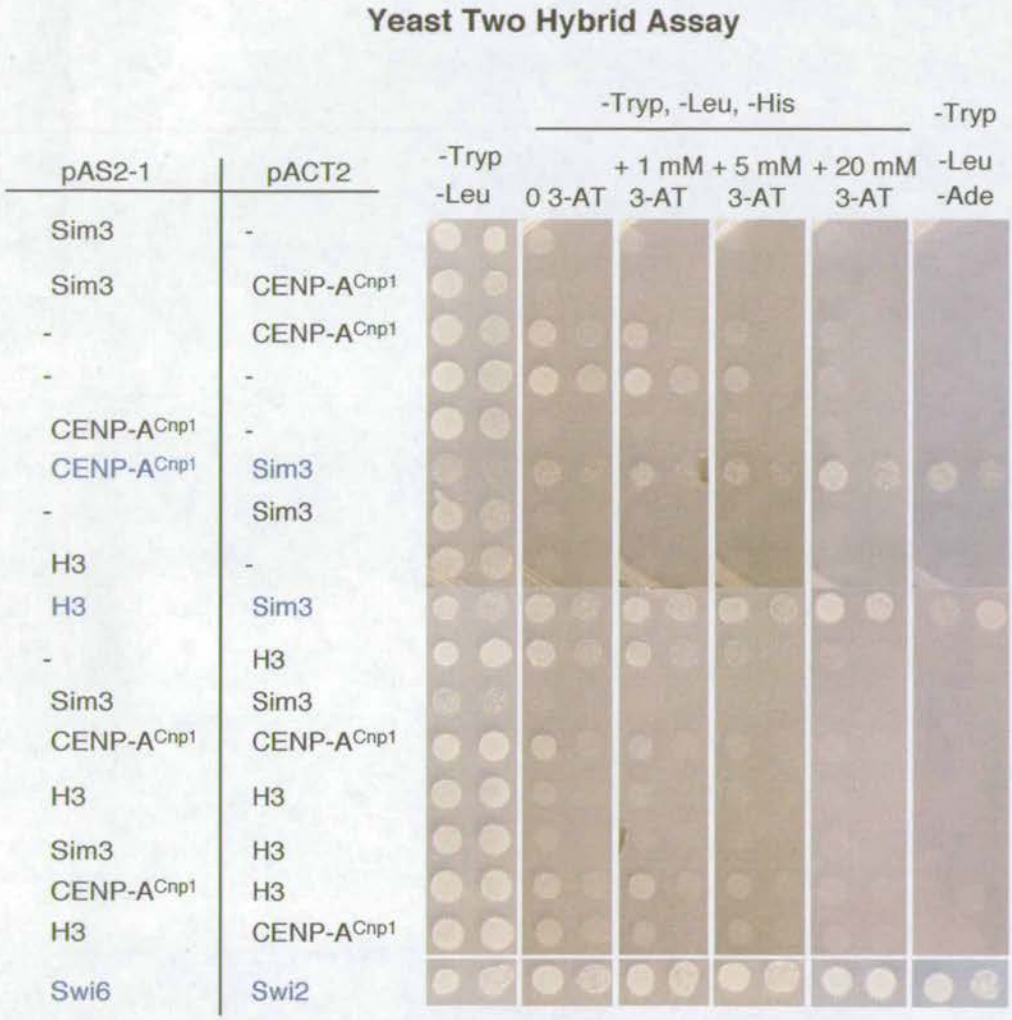


Figure 5-6. Sim3 interacts with CENP-A^{Cnp1} and histone H3 by yeast two hybrid.

All possible combinations of a pAS2-1 GAL4 DNA binding and pACT2 GAL4 activating domain plasmid were co transformed into budding yeast strain (PJ69-4A) and transformants with both plasmids were selected on medium lacking tryptophan and lacking leucine (-Tryp, -Leu). Expression of *HIS3* and *ADE6* reporter genes is dependent on the interaction between a pAS2-1 and pACT2 plasmid product. Transformants were spotted onto medium lacking leucine, tryptophan and histidine to select for *HIS*3 expression. Increasing concentrations of 3-AT (3-amino triazol) were added as an inhibitor of histidine expression to increase specificity. Expression of *ADE6* was assayed by growth on -Tryp, -Leu, -Ade. Swi6 and Swi2 were used as positive controls for interacting proteins.

5.6 Sim3 and CENP-A^{Cnp1} interact *in vivo*

To determine if Sim3 is in a complex with CENP-A^{Cnp1} *in vivo*, anti-CENP-A^{Cnp1} antibodies were used to immunoprecipitate Sim3-GFP from cell extracts (Figure 5-7). Extracts were prepared from the Sim3-GFP tagged strain and an untagged wild type control strain (1645) and proteins were immunoprecipitated using anti-CENP-A^{Cnp1} antibodies. A 'mock' IP, where extracts from the Sim3-GFP tagged strain were incubated with beads only (no antibody) was also included as a negative control. Western blot analysis revealed a band for Sim3-GFP that was not seen in the untagged extracts or in the 'mock' IP.

By yeast two hybrid analysis, Sim3 was found to interact with both CENP-A^{Cnp1} and histone H3. In addition, the *in vitro* pull-down experiments suggested a weak binding interaction between Sim3 and histone H3. To determine if Sim3 shows specificity for CENP-A^{Cnp1} binding over binding of histone H3, anti-CENP-A^{Cnp1} co-IP was performed in a *sim3*$^{+}$-GFP strain transformed with a pREP3X-H3 plasmid under the control of the *nmt1*$^{+}$ promoter. Growing these cells in the absence of thiamine for approximately 18 hours induced the over-expression of H3. These analyses show that even in the presence of an excess of histone H3, the interaction between CENP-A^{Cnp1} and Sim3-GFP was maintained to the same extent as strain transformed with empty pREP3X plasmid which was expressing normal levels of H3 (Figure 5-8). The level of H3 over-expression in extracts from untagged wild type control strain and in Sim3-GFP strain is shown by anti-H3 C-terminal western analysis (estimated levels of pREP3X-H3 over-expression from have been quantified by A. Castillo and indicate a 2.5 fold increase in protein expression). These results suggest that Sim3 binds with high affinity to CENP-A^{Cnp1} but does not exclude the possibility that Sim3 may have a weak affinity for H3 (Figure 5-8).

5.7 Over-expression of Sim3 does not lead to an increase in CENP-A^{Cnp1} at the central core

If Sim3 is required to deliver CENP-A^{Cnp1} to centromeres, then over-expression of Sim3 may affect the amount of CENP-A^{Cnp1} at the central core. To test this wild type (FY3027) and *sim3* strains were transformed with a plasmid that expresses additional Sim3 from the *nmt1*$^{+}$ promoter (pREP41X-Sim3) and Sim3 over-expression was induced in media lacking thiamine. Growth assays showed that in the wild type over-expression of Sim3 was slightly toxic to cell growth at 36°C. The over-expression of Sim3 in wild type cells did not alleviate silencing at the central core domain (no growth on media lacking arginine) nor at outer repeat heterochromatin (no growth on media lacking uracil) (Figure 5-9A). Anti-CENP-A^{Cnp1} chromatin IP was then performed on the wild type over-expressing Sim3, a control strain transformed with empty pREP41X plasmid and a wild type strain (FY1645). These analyses indicate that no increase in the level of CENP-A^{Cnp1} at *cnt* sequences was observed

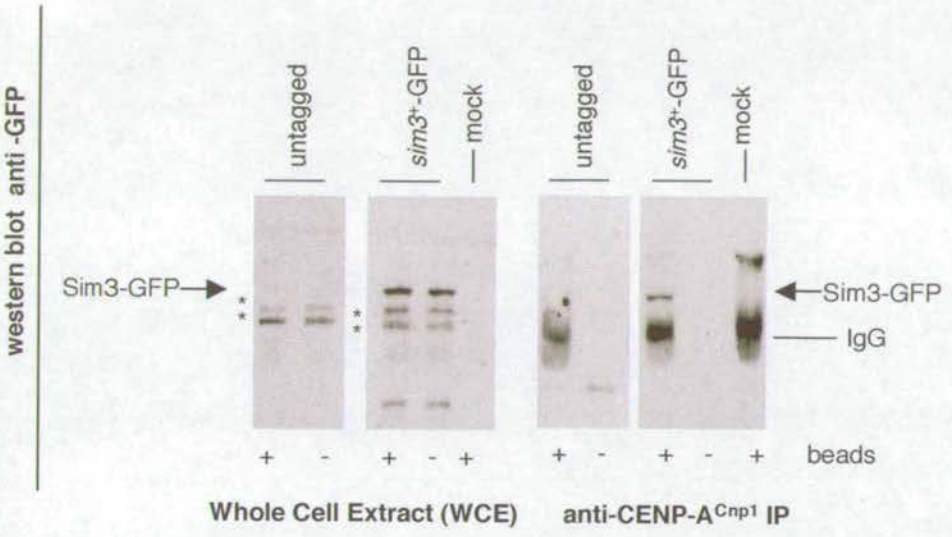


Figure 5-7. Anti-CENP-A^{Cnp1} can immunoprecipitate Sim3-GFP.

Extracts were prepared from a strain expressing Sim3-GFP and an untagged wild type strain (FY1645), which was used as a negative control. Anti-CENP-A^{Cnp1} immunoprecipitates (either + or - beads) were subjected to anti-GFP western blot. Anti-CENP-A^{Cnp1} can specifically immunoprecipitate Sim3-GFP. For mock, lysate from strain expressing Sim3-GFP was incubated with beads only (no antibody). *Cross reacting bands. IgG = immunoglobulins.

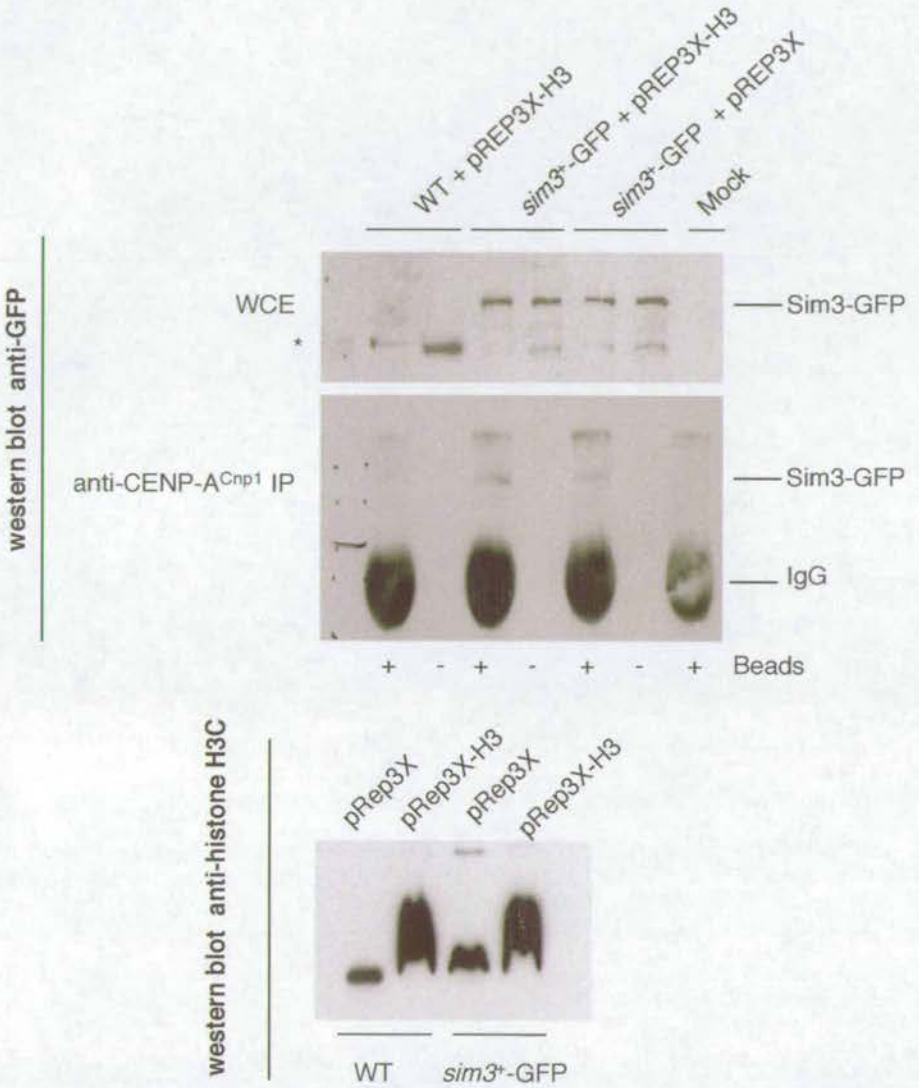


Figure 5-8. Anti-CENP-A^{Cnp1} can immunoprecipitate Sim3-GFP even in the presence of excess histone H3.

Wild type (FY1645) and strains expressing Sim3-GFP were transformed with pREP3X-H3 plasmid (and empty pREP3X plasmid) and cells were grown in the absence of thiamine to induce H3 over expression. Extracts were prepared from cells over-expressing H3 and anti-CENP-A^{Cnp1} immunoprecipitates were subjected to anti-GFP western blot. Anti-CENP-A^{Cnp1} can immunoprecipitate Sim3-GFP even in the presence of excess histone H3 (Sim3-GFP + pREP3X-H3). Amount of Sim3-GFP immunoprecipitated was similar in cells transformed with pREP3X empty plasmid only (Sim3-GFP + pREP3X). For mock, lysate from Sim3-GFP expressing strain was incubated with beads only (no antibody). Levels of histone over-expression in transformed strains is shown by anti-H3 western blot where 5 x 10^6 cells were loaded per lane. *Cross reacting bands. WCE = whole cell extract, IgG = immunoglobulins.

in cells over-expressing Sim3 and levels of CENP-A^{Cnp1} enrichment were comparable to controls (Figure 5-9B). These results show that over-expression of Sim3 does not results in the deposition of increased CENP-A^{Cnp1} at centromeres. Also, the over-expression of Sim3 in *sim3* mutants was found to re-impose silencing at the central core and does not lead to alleviation of silencing at the outer repeats (Figure 5-9A).

5.8 Sim3 is required for the deposition of newly synthesized CENP-A^{Cnp1} at centromeres

It has been shown in HeLa cells that parental CENP-A nucleosomes are randomly distributed to daughter centromeres and decrease by 50% per generation (Shelby et al., 2000). *sim3* mutants show reduced associated of bulk CENP-A^{Cnp1} with centromeres by ChIP when grown for approximately three cell divisions at 36°C. Therefore, it is possible that Sim3 is required for the delivery of newly synthesized CENP-A^{Cnp1} to centromeres. In fission yeast, the kinetochore protein Mis6 and more recently its homologue in vertebrate cells, CENP-I have been shown to affect the incorporation of new GFP tagged CENP-A at centromeres (Takahashi et al., 2000, Okada et al., 2006). To demonstrate this in fission yeast, CENP-A^{Cnp1}-GFP under the control of the *nmt1*$^{+}$ promoter was induced in the *mis6* mutant for 16 hours at 20°C, after shifting to 36°C for 4 hours (Takahashi et al., 2000). Wild type cells were shown to accumulate a single dot of CENP-A^{Cnp1}-GFP in the nucleus, *mis6* mutants however failed to accumulate a single CENP-A^{Cnp1}-GFP dot in the nucleus (Takahashi et al., 2000). The *nmt1*$^{+}$ promoter requires 16 hours for full induction, presumably due to the fact that several divisions are needed to deplete the high intra-cellular pool of thiamine (Maundrell et al., 1993). For this reason, a shorter induction time using a different promoter may be more suitable for this type of experiment. Also, as C terminal tagged CENP-A^{Cnp1}-GFP protein is not fully functional (M. Yanagida, personal communication) and it is possible that N terminal tagging of CENP-A^{Cnp1} may be more functional and represent a better alternative for detecting CENP-A^{Cnp1}.

To determine if Sim3 is required for the deposition of newly synthesized CENP-A^{Cnp1} at centromeres a different strategy was devised. A strain was constructed where GFP-CENP-A^{Cnp1} was placed under the control of the inducible invertase promoter (P*inv*). The *inv1*$^{+}$ gene of fission yeast encodes invertase. It is controlled transcriptionally by the zinc transcription factor Scr1, which is regulated by catabolite repression and can bind to three GC-rich motifs in the invertase promoter (Tanaka et al., 1998). The invertase promoter (P*inv*) is repressed when cells are grown in glucose-containing medium but can be rapidly induced (within 1-2 hours) when cells are grown in sucrose-containing medium (Iacovoni et al., 1999). Thus, the use of the P*inv* has the advantage of rapid induction protein expression compared to the *nmt1*$^{+}$ promoter, which requires 16 hours for maximum activation. Also, the P*inv* can function during cell cycle arrests, whereas the *nmt1*$^{+}$ requires the cell to

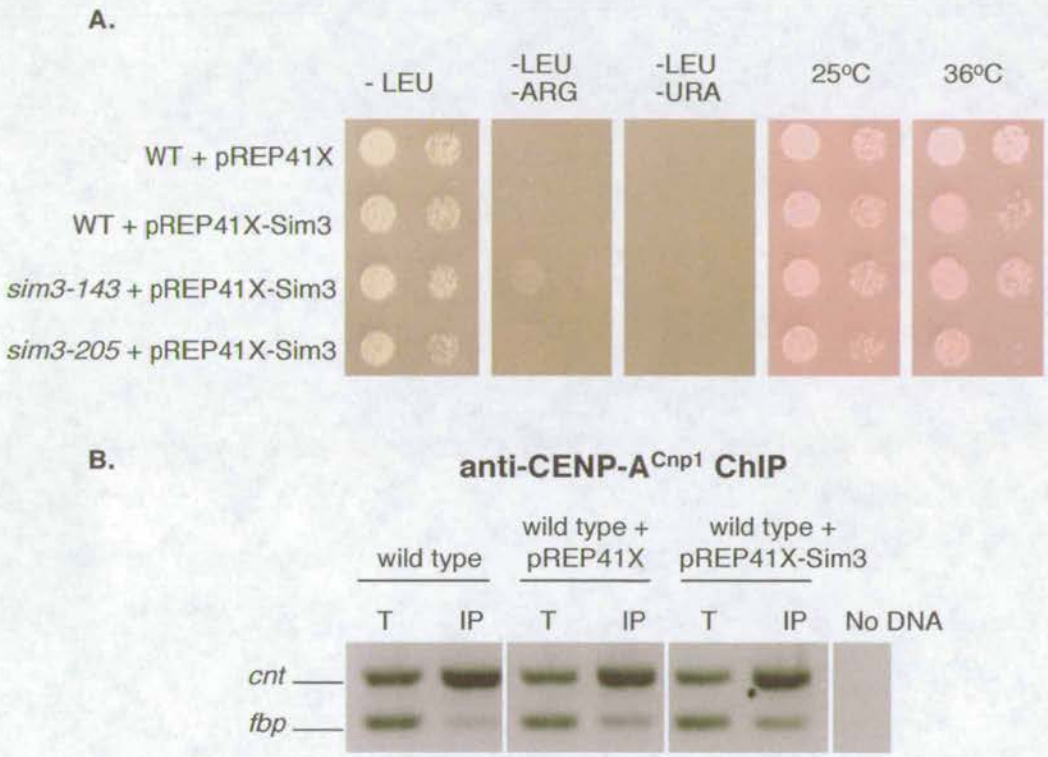


Figure 5-9. Over-expression of Sim3 does not increase the level of CENP-A^{Cnp1} at the centromere and does not lead to alleviation of silencing.

A. Wild type and *sim3* strains (3027 background) were transformed with pREP41X-Sim3 plasmid and over-expression was induced by plating on medium lacking thiamine, as protein induction is under the control of the *nmt1*$^{+}$ promoter. Over-expression of Sim3 re-imposes silencing at the central core domain (assayed by growth on -leu, -arg). Over-expression of Sim3 does not lead to alleviation of silencing at the outer repeats in wild type or *sim3* mutants (assayed by growth on -leu, -ura).

B. Chromatin immunoprecipitation with anti-CENP-A^{Cnp1} in wild type cells over-expressing Sim3 (wild type + pREP41X-Sim3) did not result in an increase in amount of CENP-A^{Cnp1} at the central core (*cnt*) compared to control strain transformed with empty plasmid (wild type + pREP41X).

undergo a number of divisions in order to deplete the cellular pool of thiamine to achieve full derepression (Maundrell et al., 1993). In addition, observations of Choi et al. (2005) demonstrated that tagged histone H3 and H4 under the control of the invertase promoter were suitable for rapid induction in fission yeast.

5.8.1 Construction of strain P*inv*-GFP-CENP-A^{Cnp1}

To construct the wild type P*inv*-GFP-CENP-A^{Cnp1} strain, the P*inv* was cloned from genomic DNA as a 2.3 kb *BamHI-PstI* fragment into a 'split URA' plasmid (constructed by Alison Pidoux, see diagram), where the *ura4*$^+$ gene is disrupted by the insertion of restriction sites. GFP-CENP-A^{Cnp1} was cloned as a *PstI-EcoR1* fragment from genomic DNA from strain FY3917 and was inserted in frame after P*inv* into 'split URA' plasmid. The plasmid was then digested with *SpeI* and *KpnI* releasing a 5 kb fragment [5'-URA-P*inv* -GFP-CENP-A^{Cnp1}-URA-3'] that was transformed into a wild type *ura4*$^+$ strain (FY96). Transformants were selected for FOA^R and positive colonies were checked for correct integration of P*inv*-GFP-CENP-A^{Cnp1} at the *ura4*$^+$ locus by PCR using a forward primer upstream of the *ura4*$^+$ 5'UTR and a reverse primer within the P*inv*. The resulting wild type P*inv*-GFP-CENP-A^{Cnp1} strain was then crossed to *sim3-143*, *sim3-205* and *mis6-302* mutants and resulting colonies were selected for temperature sensitivity by growth on phloxin at 36°C and integration of P*inv*-GFP-CENP-A^{Cnp1} at *ura4*$^+$ locus by PCR (Figure 5-10).

5.8.2 GFP-CENP-A^{Cnp1} can be induced from the invertase promoter

The invertase promoter (P*inv*) is repressed when cells are grown in glucose medium but can be rapidly induced (within 1-2 hours) when cells are grown in sucrose medium (Iacovoni et al., 1999). A time course experiment was next performed, to determine if induction of P*inv*-GFP-CENP-A^{Cnp1} followed similar dynamics to that described by Iacovoni et al. (1999). Wild type, *sim3-143* and *mis6-302* mutants containing the P*inv*-GFP-CENP-A^{Cnp1} integrated construct were first analysed at the permissive temperature of 25°C. Northern blot analysis using a probe recognizing GFP revealed that P*inv*-GFP-CENP-A^{Cnp1} is transcribed with maximal transcriptional activation 30 minutes after glucose depletion in both wild type and mutant cells (Figure 5-11A). Transcription from the invertase promoter is known to be transitory and the promoter activity is reduced again at 120 minutes after exposure to sucrose due to the conversion of sucrose to glucose (Tanaka et al., 1998). For wild type and *sim3-143* and *mis6-302* mutant strains, levels of GFP-CENP-A^{Cnp1} protein were assessed by western analysis 30, 60 and 120 minutes after induction using sheep anti-GFP antibody (Figure 5-11B). Levels of GFP-CENP-A^{Cnp1} appear to be less stable in *sim3-143* mutant after 120 minutes induction. This suggests that CENP-A^{Cnp1} protein, which possibly cannot be incorporated into the centromeres may be targeted for degradation. As levels of induced GFP-CENP-A^{Cnp1} protein were equivalent in wild type and *sim3-143* mutant strains at 60

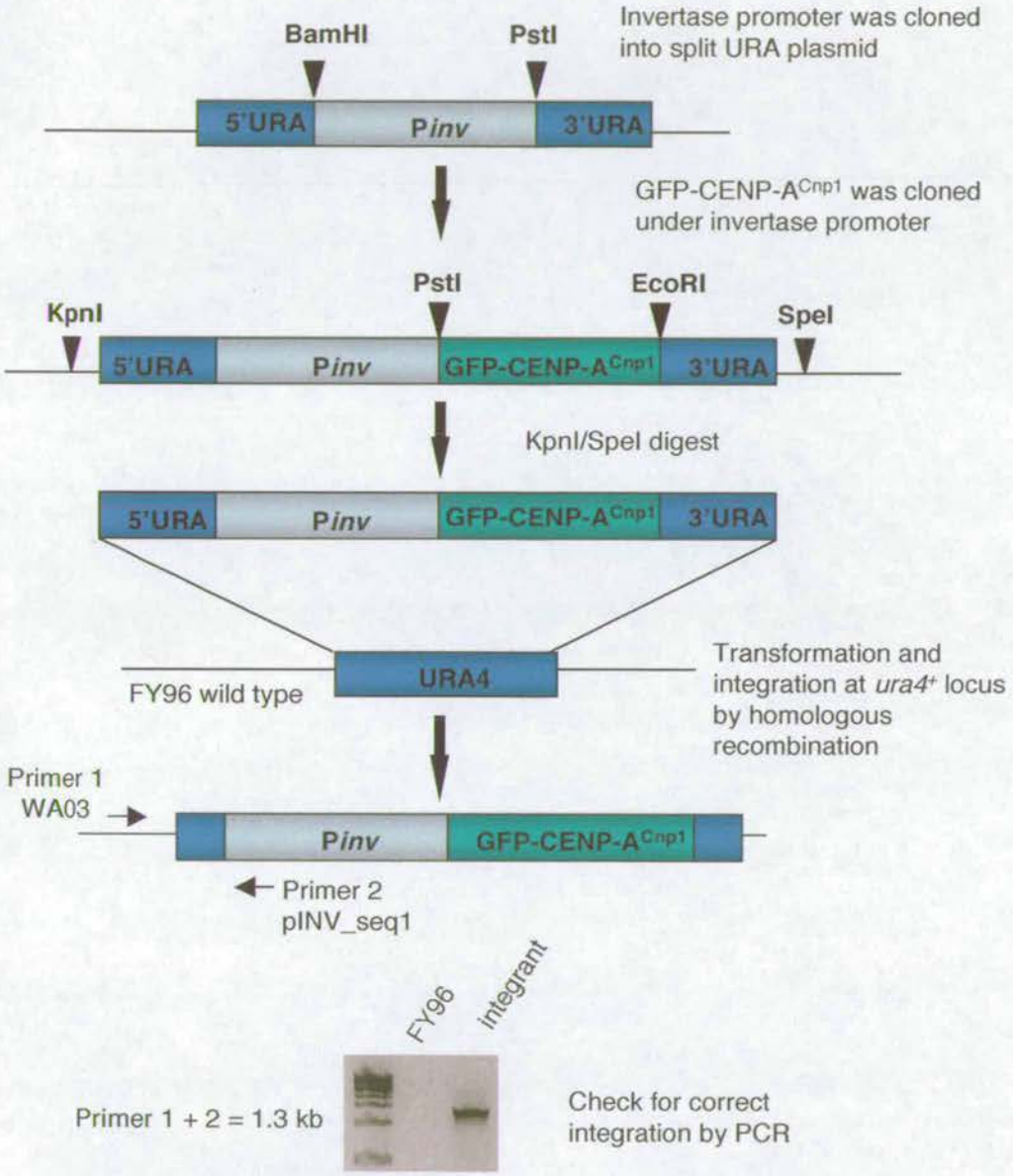


Figure 5-10. Construction of strain with GFP-CENP-A^{Cnp1} under the control of the invertase inducible promoter (P*inv*-GFP-CENP-A^{Cnp1}).

Invertase promoter (P*inv*) was cloned into 'split URA' plasmid using *BamHI* and *PstI* restriction sites. GFP-CENP-A^{Cnp1} was amplified from genomic DNA from strain FY3917 and was inserted downstream of P*inv* using *PstI* and *EcoRI* restriction sites. 5'URA-P*inv*-GFP-CENP-A^{Cnp1}-URA3' fragment was excised from plasmid using a *KpnI* and *SpeI* digest and was transformed into a wild type *ura4*$^+$ strain. Transformants were selected for FOA resistance, where P*inv*-GFP-CENP-A^{Cnp1} was integrated at the *ura4*$^+$ locus. FOA resistant colonies were checked by PCR for integration at the *ura4*$^+$ locus using a primer upstream of the *ura4*$^+$ 5'UTR (WA03) and a reverse primer within P*inv* (pINV_seq1).

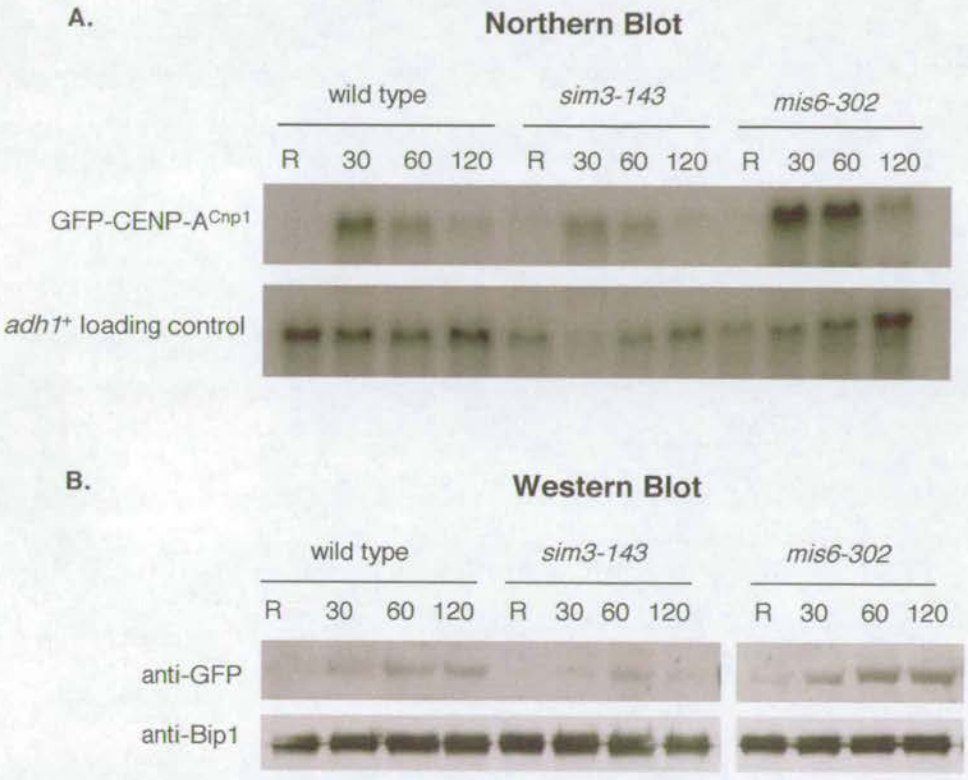


Figure 5-11. Induction of GFP-CENP-A^{Cnp1} under the control of the invertase inducible promoter at 25°C.

A. Time course of GFP-CENP-A^{Cnp1} induction in wild type, *sim3-143* and *mis6-302* and northern blot with GFP probe. GFP-CENP-A^{Cnp1} transcript was maximally induced after 30 to 60 minutes culture in PMG supplemented with 4% sucrose in both wild type and mutant cells. *adh1+* was used as a loading control. R = repressed.

B. Time course of GFP-CENP-A^{Cnp1} induction in wild type, *sim3-143* and *mis6-302* and western blot with anti-GFP antibody. Levels of induced GFP-CENP-A^{Cnp1} protein were equivalent in wild type, *sim3-143* and *mis6-302* after 60 minutes culture in PMG supplemented with 4% sucrose. Bip1 was used as a loading control. R = repressed.

minutes post-induction, all subsequent experiments were performed with 60 minutes induction in sucrose.

5.8.3 Sim3 is required for the localization of newly induced P*inv*-GFP-CENP-A^{Cnp1} by fluorescence microscopy

For each experiment, cells were cultured overnight in medium containing glucose to maintain repression of the invertase promoter. Expression from P*inv* was then induced by switching cultures to sucrose medium for 1 hour. The efficiency of the incorporation of newly synthesized GFP-CENP-A^{Cnp1} was then assessed by fluorescence microscopy for the presence of the characteristic GFP-CENP-A^{Cnp1} single bright focus of signal from the nucleus (n=200 for each condition). Analysis was carried out on both fixed cells and by live imaging to eliminate any artifacts of fixation. Un-induced samples consistently showed a GFP-CENP-A^{Cnp1} spot in 3 – 5% of cells for both wild type and mutant (not shown). After induction at 25°C, 100% of wild type cells consistently show a GFP dot in the nucleus after 60 minutes incubation in sucrose. In contrast, only 10% of *sim3-143* (10.5%) or *sim3-205* (9%) cells displayed a GFP-CENP-A^{Cnp1} signal and in most cases this signal is substantially weaker than that seen in wild type (Figure 5-12). The doubling time of wild type and *sim3* mutants is comparable at the permissive temperature, suggesting that most *sim3* cells complete the cell cycle normally. Thus, reduced GFP-CENP-A^{Cnp1} at centromeres in *sim3* mutants is not due to an indirect effect of a cell cycle delay. As a control, the incorporation of induced GFP-CENP-A^{Cnp1} was also monitored in *mis6-302* mutant cells, which had previously been shown to be required for the incorporation of CENP-A^{Cnp1} tagged with GFP on the C terminus that was induced from the *nmt1*$^{+}$ promoter (Takahashi et al., 2000). Consistent with this, no centromeric signal was observed in 86.5% cells (n=200). However, in 13.5% of cells GFP-CENP-A^{Cnp1} appeared to fill the whole nucleus and a strong GFP-CENP-A^{Cnp1} signal was observed at centromeres. The nature of this subpopulation of cells is unknown and is perhaps worth investigating further.

The incorporation of newly synthesized CENP-A^{Cnp1} was also assessed in wild type, *sim3-143*, *sim3-205* and *mis6-302* mutants at the restrictive temperature. Cells were cultured for 5 hours at 36°C and before induction of GFP-CENP-A^{Cnp1} for a further hour (a total of 6 hours at restrictive temperature). As was observed at 25°C, wild type cells were competent to incorporate new GFP-CENP-A^{Cnp1} at centromeres (99%), whereas *sim3-143* and *sim3-205* mutants showed GFP-CENP-A^{Cnp1} spots in 5% and 1% of cells respectively at 36°C. Again, *mis6-302* was unable to incorporate new GFP-CENP-A^{Cnp1} in 91.4% of cells but 8.6% of cells showed the unusual pattern described above. Western blot analysis shows that GFP-CENP-A^{Cnp1} is being induced in wild type and mutants at 36°C (Figure 5-13). In addition, cells were analysed by live imaging at to eliminate any artifacts of fixation and these analyses verified results for fixed cells analysis at both 25°C and 36°C (data not shown).

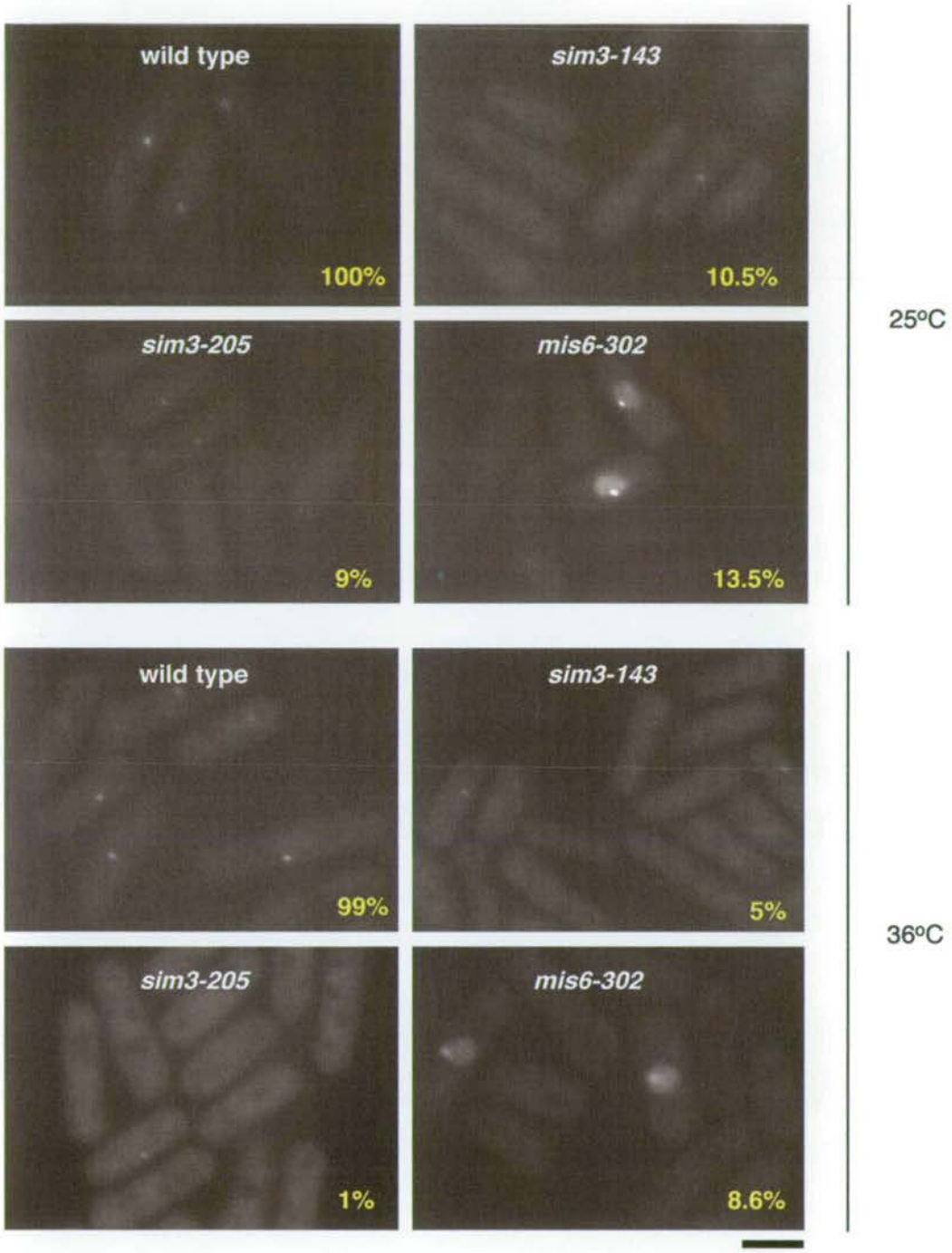


Figure 5-12. Induction of newly synthesized GFP-CENP-A^{Cnp1} in wild type, *sim3-143*, *sim3-205* and *mis6-302* mutants at 25°C and 36°C.

Strains were cultured at 25°C or shifted to 36°C for 5 hours in PMG supplemented with 10% glucose, prior to induction of P*inv*-GFP-CENP-A^{Cnp1} in PMG supplemented with 4% sucrose for a further 60 minutes at either 25°C or 36°C. Cells were fixed and analysed by fluorescence microscopy and the presence of the characteristic CENP-A^{Cnp1} signal (a single bright focus of signal per nucleus) was scored for each strain (n=200) under repressed and induced conditions and at both 25°C and 36°C. Images shown are representative of the entire population in each case. Bar, 5 μm.

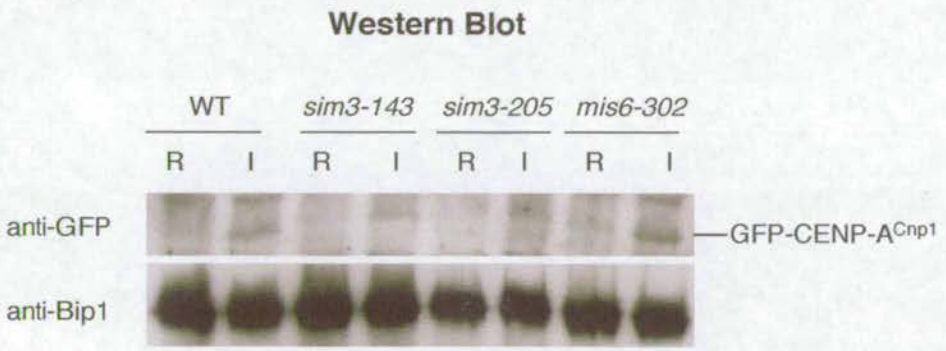


Figure 5-13. Induction of GFP-CENP-A^{Cnp1} protein in wild type, *sim3-143*, *sim3-205* and *mis6-302* after 2 hours at 36°C.

Western blot with anti-GFP antibody shows that levels of induced GFP-CENP-A^{Cnp1} protein were equivalent in wild type, *sim3-143*, *sim3-205* and *mis6-302* after 120 minutes culture in PMG supplemented with 4% sucrose. Bip1 was used as a loading control. R = repressed conditions, I = induced conditions.

5.8.4 Sim3 is required for the association of newly induced P*inv*-GFP-CENP-A^{Cnp1} with centromeres by ChIP.

To further analyse the recruitment of newly synthesized GFP-CENP-A^{Cnp1} to the centromere, anti-GFP ChIP was performed on wild type, *sim3-143* and *mis6-302* cells before and after induction of GFP-CENP-A^{Cnp1} at 25°C from the invertase promoter. In wild type cells, very little GFP-CENP-A^{Cnp1} is associated with the central domain (*cnt*) under glucose-repressed conditions. After 60 minutes induction, *cnt* is reproducibly enriched 6 fold relative to euchromatic *fbp1* (*fbp*). In *sim3-143* and *mis6-302* mutants, significantly less GFP-CENP-A^{Cnp1} was found to associate with the centromere *cnt* sequence after 60 minutes induction. The inability of *sim3* mutants to deposit newly induced GFP-CENP-A^{Cnp1} at centromeres was reproducible (Figure 5-14). These analyses indicate that in wild type cells newly made GFP-CENP-A^{Cnp1} is being deposited at the central domain chromatin of fission yeast centromere. However, although GFP-CENP-A^{Cnp1} is clearly induced in *sim3* mutants, it is inefficiently incorporated at centromeres.

5.9 Newly induced histone H3 (HA-tagged) is incorporated into chromatin in *sim3* mutants

To determine if the affect of *sim3* mutants on CENP-A^{Cnp1} is specific or if it is a general effect on other histones, the localization of newly induced histone H3 was examined. Results from the yeast two hybrid interactions and *in vitro* pull-down experiments also suggested that Sim3 might have an affinity, although weak, for H3. To test the effect of *sim3* mutations on the deposition of newly synthesized H3, a plasmid where H3 tagged with HA can be induced under the invertase promoter was utilized (P*inv*-H3-HA, described in Choi et al., 2005). Wild type, *sim3-143* and *sim3-205* strains were transformed with LEU2$^+$ P*inv*-H3-HA plasmid. *leu*$^+$ transformants were then cultured in PMG - LEU medium supplemented with 10% glucose at 25°C, in order to select for plasmid and to repress expression from P*inv*. The expression of H3-HA was then induced by incubating cells in sucrose for 2 hours. After induction, cells were fixed and stained with anti-HA antibody and the number of cells positive for incorporation of newly synthesized H3-HA was counted in wild type and mutants (n=200). For both wild type and mutants, 25% of cells in un-induced sample showed a bright nuclear signal of chromatin associated with newly induced H3-HA. This high level of background expression may be due to leaky expression from the P*inv* on the plasmid or due to variations in plasmid copy number expressed by each cell. After induction, 75% of wild type, *sim3-143* and *sim3-205* cells were positive for the incorporation of newly synthesized H3-HA into chromatin (Figure 5-15). Western analysis shows that H3-HA is efficiently and equivalently produced in both wild type and *sim3* mutant cells (Figure 5-15). These results suggest that the localization of induced HA-tagged histone H3 was unaffected by the *sim3-143* and *sim3-205* mutations and was

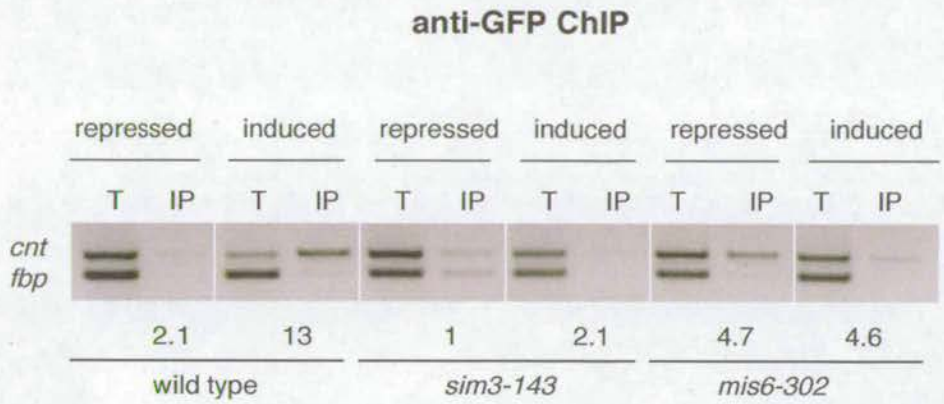


Figure 5-14. Anti-GFP chromatin immunoprecipitation on wild type, *sim3-143* and *mis6-302* strains before and after induction of P*inv*-GFP-CENP-A^{Cnp1} at 25°C.

Strains were cultured at 25°C under repressive conditions prior to induction of P*inv*-GFP-CENP-A^{Cnp1} for 60 minutes in sucrose medium (induced) or continually cultured under repressive conditions (repressed). *cnt* enrichment is measured relative to the *fbp* euchromatic control and normalised to the input PCR.

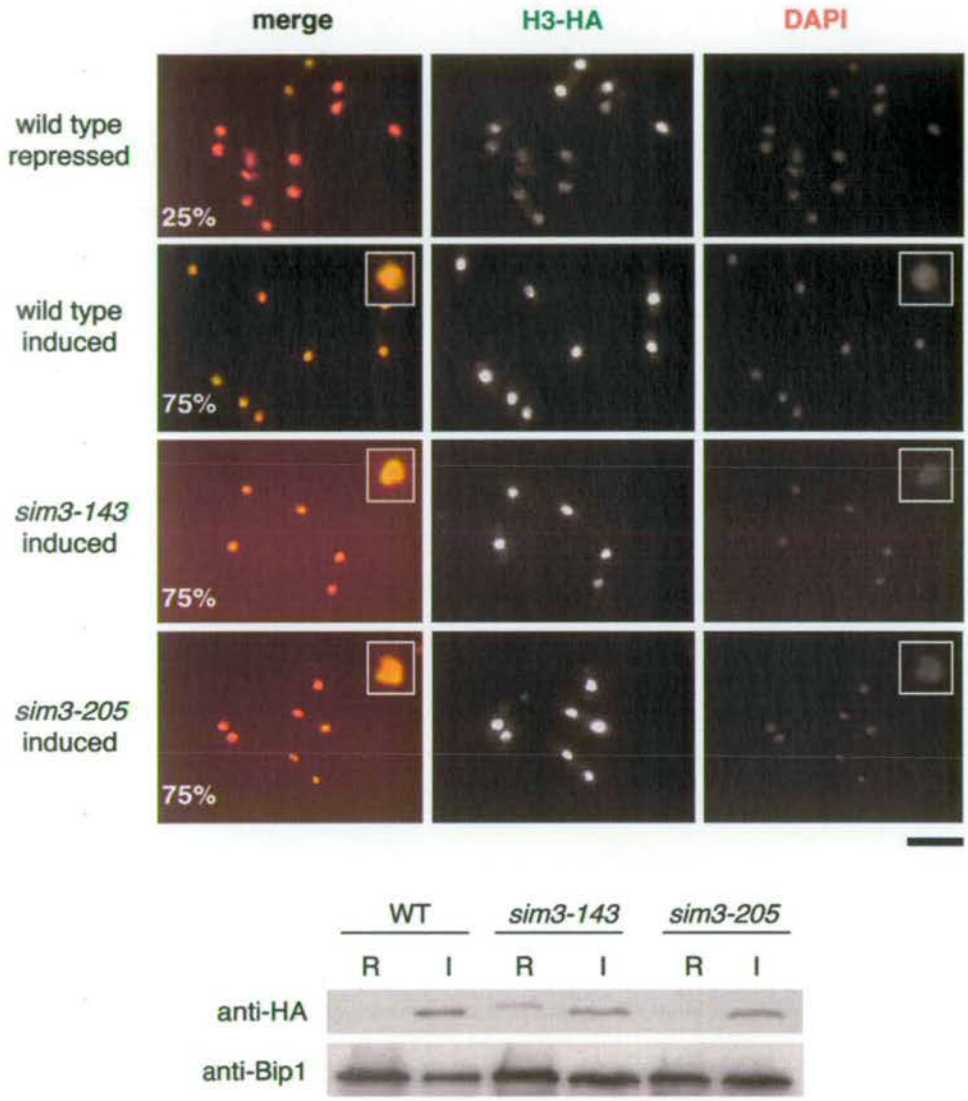


Figure 5-15. Induction of newly synthesized H3-HA in wild type and *sim3* mutants at 25°C.

Wild type and *sim3* mutants were transformed with P*inv*-H3-HA plasmid and expression of H3-HA was induced by culturing cells in PMG media lacking leucine (to select for plasmid) and supplemented with 4% sucrose. Cells were grown in PMG -LEU supplemented with 10% glucose to repress H3-HA expression. Repressed or induced cells were fixed and stained with anti-HA antibody (green) and DNA was stained with DAPI (red). Under repressed conditions in both wild type and *sim3* mutants (not shown) 25% of cells (n=200) were positive for H3-HA expression, where H3-HA signal fills the entire nucleus and staining pattern tightly overlaps with DAPI staining. Both wild type and *sim3* cells showed a H3-HA signal in 75% of cells analysed under induced conditions (n=200). Levels of H3-HA induction in wild type and *sim3* mutants was assessed by western blot using anti-HA antibody. R = Repressed conditions, I = Induced conditions. Bip1 is used as a loading control. Bar, 10 μm.

identical to that observed in wild type. Therefore *sim3-143* and *sim3-205* do not affect the deposition of newly produced histone H3 and are specifically required for the deposition of newly synthesized histone H3 variant CENP-A^{Cnp1} at centromeres.

5.10 CENP-A^{Cnp1} can be deposited at G2 phase of the cell cycle

From studies from human cells, *Drosophila*, fission yeast and plants is was proposed that CENP-A might be deposited by replication-coupled mechanisms in S phase and replication-independent mechanisms in the G2 phase of the cell cycle (Shelby et al., 1997, Sullivan and Karpen, 2001, Takahashi et al., 2005, Lermontova et al., 2006). In fission yeast, the GATA-like transcription factor Ams2 was shown to play a role in the replication coincident pathway, which may not necessarily be coupled to replication (Takahashi et al., 2005). It is likely that Mis6 plays a dual role in both S phase and G2 loading of CENP-A^{Cnp1} (Takahashi et al., 2005). However, other factors that contribute to the replication-dependent or replication-independent deposition of CENP-A in fission yeast and higher eukaryotes are not known.

A previous study by Takahashi et al. (2005) utilizing the *cdc25-22* mutant demonstrated that fission yeast CENP-A^{Cnp1} can be deposited during the G2 phase. Cdc25 phosphatase is required to activate the cyclin dependent kinase Cdc2 and thereby promotes G2/M transition (Miller et al., 1991). Takahashi et al. (2005) demonstrated that newly synthesized CENP-A^{Cnp1}-GFP expressed from the *nmt1*$^+$ promoter localizes to centromere-like dots in the nucleus in *cdc25-22* cells that were arrested in G2 (4 hours at 36°C). These observations demonstrate that ectopically induced tagged CENP-A^{Cnp1} protein can be loaded onto centromeres at interphase (Takahashi et al., 2005). However, as referred to above, in order to achieve full induction of the *nmt1*$^+$ promoter, cells were incubated for 15 hours without thiamine at the permissive temperature before switching to the restrictive temperature. The use of GFP-CENP-A^{Cnp1} expressed from the invertase promoter circumvents the problem of the long induction time of the *nmt1*$^+$ promoter and is again a more suitable alternative to use when examining the localization of a newly synthesized protein.

To confirm the existence of a CENP-A^{Cnp1} loading pathway in G2 using the more rapid and convenient inducible invertase system, the P*inv*-GFP-CENP-A^{Cnp1} was crossed with a *cdc25-22* strain. *cdc25-22* P*inv*-GFP-CENP-A^{Cnp1} cells were first arrested in G2 by incubation for 3 hours at 36°C before induction of newly synthesized GFP-CENP-A^{Cnp1} in sucrose for 1 hour. After a total of 4 hours at 36°C, cells were fixed and processed for fluorescence microscopy or ChIP. Consistent with the *nmt1*$^+$ promoter induced CENP-A^{Cnp1} that can be deposited during the G2 phase, under these conditions ectopically induced GFP-CENP-A^{Cnp1} protein under the invertase promoter was loaded onto centromeres in 99% of G2 arrested *cdc25-22* cells (Figure 5-16A). As approximately 80% of *S. pombe* cells in a cycling population are in

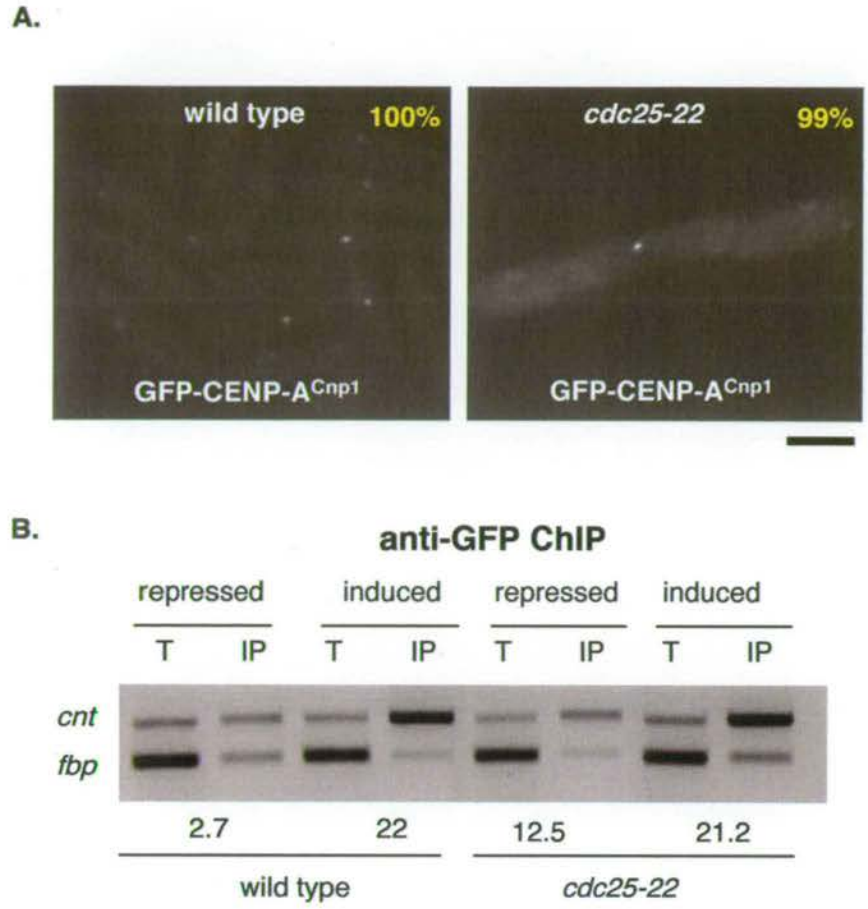


Figure 5-16. Replication independent loading of GFP-CENP-A^{Cnp1} in G2.

A. Immuno-fluorescence showing GFP-CENP-A^{Cnp1} is localised as a spot to the nucleus in wild type and *cdc25-22* mutant cells. Cells were cultured for 2 hours at restrictive temperature before induction of new GFP-CENP-A^{Cnp1} for 2 hours. Bar, 3 μm

B. Anti-GFP chromatin immunoprecipitation showing recruitment of GFP-CENP-A^{Cnp1} to the central core after induction under invertase promoter in wild type and *cdc25-22* mutants at 25°C.

G2, experiments described in 5.8.3 also provide evidence that newly synthesized GFP-CENP-A^{Cnp1} can be loaded at centromeres outside of S phase.

ChIP with anti-GFP antibody was also carried out on uninduced and induced *cdc25-22* cells, showing that, when induced, newly synthesized GFP-CENP-A^{Cnp1} was enriched at the central core (*cnt*) relative to *fbp1* (Figure 5-16B). In addition, GFP-CENP-A^{Cnp1} was found to be enriched to the same extent in both wild type and *cdc25-22* strains. Thus, newly induced CENP-A^{Cnp1} protein can be successfully loaded onto the centromere of cells, which were blocked in G2 phase of the cell cycle.

5.11 Sim3 is required for the replication independent deposition of CENP-A^{Cnp1} in G2

The above analysis demonstrates that CENP-A^{Cnp1} can associate with centromeres during G2 phase. To determine whether Sim3 is required for the replication independent incorporation of GFP-CENP-A^{Cnp1}, P*inv*-GFP-CENP-A^{Cnp1} was introduced into *sim3 cdc25-22* double mutants by crossing. In order to create *sim3 cdc25-22* double mutants and as both *sim3* and *cdc25-22* are temperature sensitive mutations, strains were crossed and resulting asci were dissected. Non parental ditype (NPD) tetrads with two wild type and two ts spores, were identified from the cross, where ts was assayed by growth on phloxin at 36°C. NPD ts strains are double mutants for both *sim3* and *cdc25* and the presence of P*inv*-GFP-CENP-A^{Cnp1} was confirmed by PCR. *sim3-143 cdc25-22* and *sim3-205 cdc25-22* double mutants strains were also sequenced to ensure the presence of *sim3* point mutations. Cells were clearly *cdc25-22* since at 36°C they arrested with a characteristic long cell phenotype indicative of cell cycle arrest.

To test whether GFP-CENP-A^{Cnp1} can be loaded at centromeres during G2 in cells with defective Sim3, the *cdc25-22* mutant and *sim3-143 cdc25-22* and *sim3-205 cdc25-22* double mutants were grown overnight at 25°C in PMG supplemented with 10% glucose. Cells were then shifted to 36°C for 3 hours and GFP-CENP-A^{Cnp1} was induced by switching cells to PMG media containing 4% sucrose for 1 hour. After a total of 4 hours at 36°C, cells were fixed, DAPI stained to identify the nucleus and analysed by fluorescent microscopy for the presence of a GFP-CENP-A^{Cnp1} dot at centromeres (n=200 for each strain) (Figure 5-17). Again, in agreement with the previous experiment, newly induced GFP-CENP-A^{Cnp1} protein was loaded onto centromeres in 99% of G2 arrested *cdc25-22* cells. In striking contrast, 0% of *sim3-143 cdc2-225* and 0% of *sim3-205cdc25-22* double mutants were able to incorporate newly synthesized GFP-CENP-A^{Cnp1} after induction under restrictive conditions. The GFP-CENP-A^{Cnp1} protein was being stably produced under these conditions as demonstrated by western analysis (data not shown). These results indicate that the NASP related protein Sim3 is required for the efficient replication independent deposition of CENP-A^{Cnp1} at fission yeast centromeres.

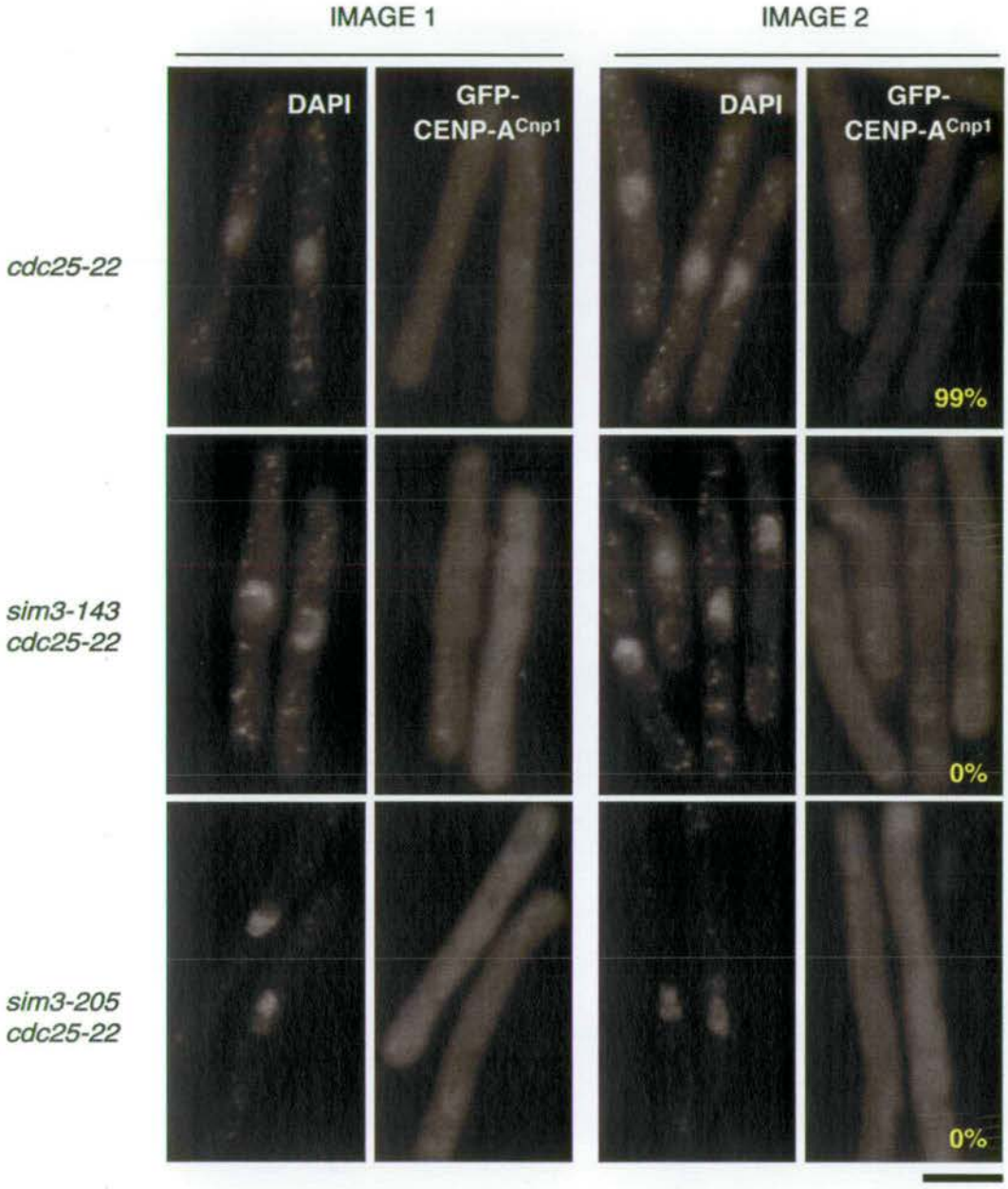


Figure 5-17. Induction of newly synthesized GFP-CENP-A^{Cnp1} in *cdc25-22*, *cdc25-22 sim3-143* and *cdc25-22 sim3-205* mutants at 36°C.

Strains were cultured at 25°C and shifted to 36°C for 3 hours in PMG supplemented with 10% glucose, prior to induction of P*inv*-GFP-CENP-A^{Cnp1} in PMG supplemented with 4% sucrose for a further 60 minutes 36°C. Cells were fixed, DAPI stained and analysed by fluorescent microscopy for the presence of the characteristic CENP-A^{Cnp1} signal (a single bright focus of signal per nucleus) was scored for each strain (n=200) under repressed and induced conditions. After 4 hours culture at 36°C, *cdc25-22* cells appear characteristically long due to cell cycle block at G2. IMAGE1 and IMAGE 2 are duplicate samples from the same experiment. Bar 5 μm.

DISCUSSION

In this chapter, the role of Sim3 in the delivery of CENP-A^{Cnp1} to the centromere was investigated. *sim3* mutants alleviate silencing at the central core domain, where the histone H3 variant CENP-A^{Cnp1} and the kinetochore are assembled and mutations in *sim3* result in defects in centromere function and chromosome segregation (described in chapter 4). In this chapter, *sim3* mutants were found to have reduced CENP-A^{Cnp1} associated with centromeres by immuno-staining and by ChIP. Reciprocal to this, *sim3* mutants were found to have increased histone H3 associated with the central domain of the centromere. Sim3 was found to bind to CENP-A^{Cnp1} both *in vitro* and *in vivo*, and may have a weak affinity for H3 *in vitro*. Sim3 is required for the association of newly synthesized GFP-CENP-A^{Cnp1} with centromeres. In particular, Sim3 is required for the association of newly synthesized GFP-CENP-A^{Cnp1} with centromeres during G2 phase of the cell cycle. From the data presented in this chapter, it is proposed that Sim3 is acting as a chaperone for CENP-A^{Cnp1} and facilitates the replication independent assembly of CENP-A^{Cnp1} at fission yeast centromeres.

Sim3 is required for the association of endogenous CENP-A^{Cnp1} with the centromere

The association of CENP-A^{Cnp1} with centromeres was found to be reduced at both permissive and restrictive temperatures in *sim3* mutants by immuno-fluorescence staining and by ChIP. Some nucleosomes containing histone H3 have been detected in the central core domain (Cam et al., 2005), but they are underrepresented in this region relative to the rest of the genome. In *sim3* mutants however, chromatin IP with anti-H3 antibody revealed that H3 association with the central core region is increased. Thus in *sim3* mutants it appears that the amount of CENP-A^{Cnp1} at centromeres is reduced, and reciprocally, the amount of histone H3 at the central core is increased. A similar result was reported in flies, where examination of chromatin fibers from CENP-A^{CID} RNAi-treated cells led to an increase in H3 stained regions at the centromere, also suggesting that the composition of centromeric chromatin is plastic (Blower et al., 2002). Sim3 is not merely affecting the amount of CENP-A^{Cnp1} being produced in the cell, as levels of myc-tagged CENP-A^{Cnp1} expressed under its endogenous promoter, were stable and comparable to wild type in *sim3* mutants at permissive and restrictive temperature. These results suggest a role for Sim3 in facilitating the assembly of CENP-A^{Cnp1} chromatin. It is also possible that Sim3 may play a role in regulating the association of H3 with the central core.

Alternatively, Sim3 may function by affecting the exchange of histone H3 with CENP-A^{Cnp1} during chromatin assembly. Observations in *Drosophila* and human cells have shown that H3 is replaced by the histone variant H3.3 shortly after transcription is induced (Ahmad and Henikoff, 2002, Janicki et al., 2004, Schwartz and Ahmad, 2005). The deposition of H3.3 at transcriptionally active regions of the genome may be mediated by HIRA complex that

facilitates DNA synthesis independent nucleosome assembly (Ray-Gallet et al., 2002, Tagami et al., 2004). Similarly, the SWI/SNF-related remodelling factor, SWR1, has been shown to catalyze histone variant exchange *in vitro* by displacing H2A and replacing with the variant H2A.Z (Mizuguchi et al., 2004). It is possible that Sim3 may be acting in a similar manner to facilitate that replacement of histone H3 with CENP-A^{Cnp1} at centromeres. A model for the possible role of Sim3 in histone exchange at the centromere is described in detail in Discussion chapter (Model 2).

Sim3 interacts directly with CENP-A^{Cnp1} and may act as a CENP-A^{Cnp1} chaperone

Consistent with the proposed role of Sim3 as a CENP-A^{Cnp1} chaperone, Sim3 and CENP-A^{Cnp1} were shown to interact directly *in vitro* and CENP-A^{Cnp1} immunoprecipitates with Sim3-GFP *in vivo*. Mutant Sim3 proteins were very inefficient at CENP-A^{Cnp1} binding *in vitro*. One possibility is that reduced efficiency of CENP-A^{Cnp1} binding results in the reduced association of CENP-A^{Cnp1} with centromeres, as CENP-A^{Cnp1} is no longer available in a ready state for incorporation into chromatin and it would be of interest to examine if this is the case *in vivo* by determining if the association of CENP-A^{Cnp1} with Sim3 is altered in *sim3* mutants at permissive and restrictive temperatures.

As shown in chapter 4, Sim3 is an abundant protein that localizes to the nucleus throughout the cell cycle and analyses of Sim3 and CENP-A^{Cnp1} co-staining by microscopy and ChIP were unable to detect the association of Sim3 with the centromere. This suggests that Sim3 may be transiently associated with centromeric chromatin at an undetectable level or only at a certain stage of the cell cycle. This also implies that Sim3 may need to interact with a yet unknown protein, factor X, to which it hands over CENP-A^{Cnp1} for targeting to the centromere in order to exert its effects of centromeric CENP-A^{Cnp1} association. It is possible that factor X may be a chromatin-remodelling factor that is associated with centromeric chromatin and directly deposits CENP-A^{Cnp1} at centromeres (see Models 1A and 1B in Discussion chapter).

In addition, Sim3 was also found to have a weak affinity for histone H3 *in vitro*. The interaction between both Sim3 and CENP-A^{Cnp1} and Sim3 and H3 were confirmed by yeast two-hybrid analysis. Interestingly, the mammalian Sim3 homologue NASP has been shown to be part of both the H3.1-CAF1 and H3.3-HIRA complexes in HeLa cells (Tagami et al., 2004), suggesting that Sim3 may also be complexed to H3, as well as CENP-A^{Cnp1}. It is possible that CENP-A^{Cnp1} and H3 compete for Sim3 binding. However, as the co-IP between CENP-A^{Cnp1} and Sim3-GFP is maintained even in the presence of excess histone H3, this suggests that although Sim3 may bind to H3, it has a greater affinity for CENP-A^{Cnp1}, which is much less abundant in the cell.

Sim3 is required for the deposition of newly synthesized GFP-CENP-A^{Cnp1}

In fission yeast, the kinetochore protein Mis6 and its homologue in vertebrate cells, CENP-I, have been shown to affect the incorporation of new GFP tagged CENP-A at centromeres (Takahashi et al., 2000, Okada et al., 2006). In addition, fission yeast Mis16 and Mis18 and homologues in vertebrates RbAp46-RbAp48 are required for the association of CENP-A with centromeres (Hayashi et al., 2004). However, a direct interaction between these proteins with CENP-A has not been demonstrated. To determine if Sim3 is required to deliver newly synthesized CENP-A^{Cnp1} to the centromere, a strain was constructed where GFP-CENP-A^{Cnp1} could be quickly induced under the control of the invertase promoter (P*inv*-GFP-CENP-A^{Cnp1}). In wild type, *sim3-143* and *mis6-302* cells P*inv*-GFP-CENP-A^{Cnp1} was induced after 60 minutes. However, newly synthesized GFP-CENP-A^{Cnp1} appears to be slightly unstable at 120 minutes in *sim3-143*, compared to wild type or *mis6-302*. In budding yeast, it has been shown that proteolysis of excess CENP-A^{Cse4p} regulates the level of CENP-A^{Cse4p} available for targeting to the centromere (Collins et al., 2004). In addition, the double knockdown of the human Mis16 homologues RbAp46/RbAp48, which are required for CENP-A localization at centromeres in HeLa cells, resulted in reduced stability of the CENP-A protein but not CENP-A transcript (Hayashi et al., 2004). Thus, it is possible that in *sim3* mutants, newly synthesized GFP-CENP-A^{Cnp1} is not efficiently incorporated at the centromere and must be degraded. This is inconsistent however, with the fact that endogenous levels of myc-tagged CENP-A^{Cnp1} and over-expression of pREP3X-CENP-A^{Cnp1} did not show this instability in *sim3* mutants at either 25°C or 36°C.

sim3 mutants failed to localize newly synthesized GFP-CENP-A^{Cnp1} to centromeres by immuno staining and by anti-GFP ChIP. *mis6-302* mutants were also compromised in the targeting of newly synthesized GFP-CENP-A^{Cnp1} to the central core domain by IF as previously published (Takahashi et al., 2000) and this result was confirmed by ChIP. These results implicate Sim3 in the delivery of GFP-CENP-A^{Cnp1} to the centromere and strengthen further the proposal that Sim3 is a CENP-A^{Cnp1} chaperone. Also, there could be mechanisms in place to maintain CENP-A^{Cnp1} at centromeres and Sim3 may be involved in making sure that CENP-A^{Cnp1} is retained at centromeres once it is deposited. It is also possible that Sim3 contributes to both establishing and maintaining CENP-A^{Cnp1} chromatin.

Fission yeast CENP-A^{Cnp1} can be incorporated at centromeres during interphase and Sim3 is required for this process

Studies in human, flies, fission yeast and in plants have shown that CENP-A may be deposited during replication in S phase, in a similar manner to the other core histones, and may also be deposited by replication independent mechanisms in the G2 phase of the cell cycle (Ahmad and Henikoff, 2001, Shelby et al., 2000, Takahashi et al., 2005, Lermontova et

al., 2006). Firstly, the presence of a loading pathway for GFP-CENP-A^{Cnp1} in G2 was confirmed by fluorescence microscopy and ChIP by inducing newly synthesized GFP-CENP-A^{Cnp1} in a strain carrying a *cdc25-22* mutation blocked in G2. To assess if Sim3 is required for the replication independent incorporation of *Pinv*-GFP-CENP-A^{Cnp1}, *sim3-143cdc25-22* and *sim3-205cdc25-22* double mutants were created. When blocked in G2, *sim3cdc25-22* double mutants could no longer target newly synthesized GFP-CENP-A^{Cnp1} to the centromere. These results implicate Sim3 in the delivery of GFP-CENP-A^{Cnp1} to the centromere in pathway that is independent of replication in S phase, but does not exclude the possibility that Sim3 is playing a role in CENP-A^{Cnp1} chromatin assembly at other stages of the cell cycle. It would be interesting to test the effect of *sim3* mutants on CENP-A^{Cnp1} deposition in S phase by blocking with hydroxyurea or in G1 phase using the *cdc10* mutation. Possible mechanisms of CENP-A^{Cnp1} chromatin assembly in interphase may involve stripping out of deposited H3 and direct replacement with CENP-A^{Cnp1} or by filling gaps left for CENP-A^{Cnp1} in the chromatin after DNA replication has occurred and are described in detail in Discussion chapter (Model 2 and 3).

As discussed above, the GATA-like transcription factor Ams2 was shown to be responsible for the replication coupled pathway and it is likely that Mis6 plays a dual role in both S phase and G2 deposition of CENP-A (Takahashi et al., 2005). Evidence for this comes from the fact that when *mis6* gene is continually inactivated from G1 to G2 phase, unequal segregation occurs in the next M phase, whereas inactivation of *mis6* after the completion of S phase did not result in mis-segregation in the following M phase (Saitoh et al., 1997). Combining the *mis6* ts mutant with and *ams2Δ* results in cells which exhibit unequal chromosome segregation in the first M phase after *mis6* inactivation, providing evidence that Mis6 contributes at least partly to the back-up G2 deposition of CENP-A^{Cnp1} (Takahashi et al., 2005). The *mis6-302 sim3-143* double mutant does not show any growth impairment compared to the single mutant (Pidoux et al., 2003), which also implies that Sim3, like Mis6, is acting in the replication-independent loading pathway. The GATA-like factor Ams2 has been implicated in the deposition of CENP-A^{Cnp1} in S phase in fission yeast (Takahashi et al., 2005). Transformation of a multi-copy plasmid of *ams2*$^{+}$ failed to complement *sim3* temperature sensitivity (data not shown), indicating the restoration of replication dependent loading of CENP-A^{Cnp1} is not sufficient to rescue *sim3* mutants and the replication independent loading of CENP-A^{Cnp1} appears to be vital for cell viability. The role of this G2 deposition pathway, which follows DNA replication may be to act as a safeguard to ensure the CENP-A nucleosomes are correctly assembled to facilitate proper assembly of the kinetochore before entering the subsequent mitosis.

In summary, data from this chapter is consistent with a role for Sim3 as a CENP-A^{Cnp1} chaperone. Sim3 is a highly conserved protein and *sim3* mutations were found to occur in conserved residues. The roles of Sim3 homologues in CENP-A chromatin assembly in

higher eukaryotes have not been determined. This prompted an investigation into the role of the *Drosophila melanogaster* Sim3 homologue, DmNASP, in determining CENP-A^{CID} localization, which is discussed in chapter 6.

CHAPTER 6

ROLE OF *DROSOPHILA MELANOGASTER* DmNASP IN CENP-A^{CID} CHROMATIN ASSEMBLY

INTRODUCTION

In chapter 5, the ability of the nuclear protein Sim3 to act as a CENP-A^{Cnp1} chaperone in fission yeast was demonstrated. Moreover, Sim3 mediates the assembly of CENP-A^{Cnp1} chromatin at centromeres in a manner that is independent of replication. Sim3 is a highly conserved protein and *sim3* mutations were found to occur in conserved residues. Sim3 shows similarity to a pair of polypeptides in *Xenopus laevis* termed N1/N2 and to mammalian nuclear autoantigenic sperm protein (NASP), both of which have been shown to act as histone chaperones (Kleinschmidt et al., 1985; Kleinschmidt et al., 1990, Richardson et al., 2000, Alekseev et al., 2003). Histone chaperones are negatively charged molecules that keep the highly positively charged histones in a protected state, safe from the negatively charged DNA and prevent histones from precipitating (reviewed Haushalter and Kadonaga, 2003). In this way, histone chaperones allow the assembly of chromatin to occur in a precise and co-ordinated manner. Other mammalian histone chaperones include the H2A-H2B binding nucleosome assembly protein 1 (NAP1) (Ito et al., 1996, Ishimi et al., 1987), the chromatin assembly factor (CAF1) which coordinates nucleosome remodelling with DNA replication (Smith and Stillman, 1989) or repair (Gaillard et al., 1996) and HIRA (histone cell cycle regulation defective) which has been shown to act as a chaperone for DNA-synthesis-independent nucleosome assembly (Ray-Gallet et al., 2002).

In *Xenopus laevis* oocytes, non-chromatin bound histones H3 and H4 are found in association with N1/N2 (Kleinschmidt et al., 1985). The histones H3 and H4 present in the N1/N2 complex can be transferred to DNA *in vitro*, measured as supercoil induction into a relaxed circular plasmid DNA, which suggests a role for this highly abundant nuclear protein in chromatin assembly (Kleinschmidt et al., 1985; Kleinschmidt et al., 1990). Mammalian NASP was first described in as a cell cycle regulated, linker histone H1 binding protein (Richardson et al., 2000). NASP occurs in two major forms: testicular NASP (tNASP) found in gametes and in the embryo and a shorter version called somatic NASP (sNASP) which is found in all rapidly dividing somatic cells and arises from alternative splicing (Richardson et al., 2000). It is proposed that tNASP functions to store histones in the early events of spermatogenesis when DNA replication is uncoupled from histone synthesis (O'Rand et al., 1992). In addition, it has been demonstrated, using *in vitro* DNA supercoiling assays, that histone H1-tNASP complexes can transfer H1 histones to DNA (Alekseev et al., 2003). Knockdown of NASP in HeLa cells results in cells that are unable to replicate their DNA and cannot progress through the cell cycle and the NASP$^{-/-}$ null

mutation results in embryonic lethality in mice (Richardson et al., 2006). In addition, over-expression of tNASP, but not sNASP, delays the progression of cells through the G1/S stage of the cell cycle (Alekseev et al., 2003). More recently, both tNASP and sNASP were found to co-purify with the CAF1-histone H3.1 and HIRA-histone H3.3 complexes, which facilitate replication dependent and independent H3 chromatin assembly respectively (Tagami et al., 2004). Thus, Sim3 homologues appear to perform chaperone-like functions in other organisms, however it is possible that Sim3 homologues may play different roles in different cell types and organisms.

A single Sim3/NASP homologue, designated DmNASP, was found to be present in the genome of the fruit fly *Drosophila melanogaster*. The domain organisation of the *D. melanogaster* centromere resembles those of both human and *S. pombe* centromeres, where the chromatin containing the CENP-A homologue CID (centromere identifier) is found embedded in centric heterochromatin (Blower et al., 2002). Functional CENP-A^{CID} is required for accurate mitotic segregation of chromosomes, for cell cycle progression and the assembly of the kinetochore in flies (Blower and Karpen, 2001). Knockdown of gene expression using double stranded RNA-mediated interference has been optimised in the *Drosophila melanogaster* tissue culture Schneider (S2) cell line (Maiato et al., 2003) and provides a convenient and efficient method to investigate the function of a gene of interest. This chapter describes an investigation into the role of DmNASP in the fly S2 cell line. To determine if the role of Sim3 is conserved in more complex eukaryotes, RNAi was used to deplete DmNASP in the S2 tissue-culture cell line and the localisation of CENP-A^{CID} was determined. In addition, the ability of DmNASP to bind histones was assessed.

RESULTS

6.1 CG8322 is the *Drosophila melanogaster* homologue of fission yeast Sim3 and mammalian NASP

From analysis of BLAST searches carried out in chapter 4, a single Sim3 homologue was identified in the *Drosophila melanogaster* genome, designated DmNASP (CG8322). Fission yeast Sim3 shows approximately 24% identity to DmNASP. DmNASP contains two putative histone binding domains, based on homology to mammalian NASP and *Xenopus laevis* N1/N2 and in addition a C terminal coiled-coil domain which may facilitate protein-protein interactions (Figure 6-1).

6.2 Production of anti-DmNASP N terminal and C terminal antibodies

To determine the localisation of DmNASP and in order to visualise DmNASP protein by western blot anti-DmNASP antibodies were generated. To raise antibodies to DmNASP,

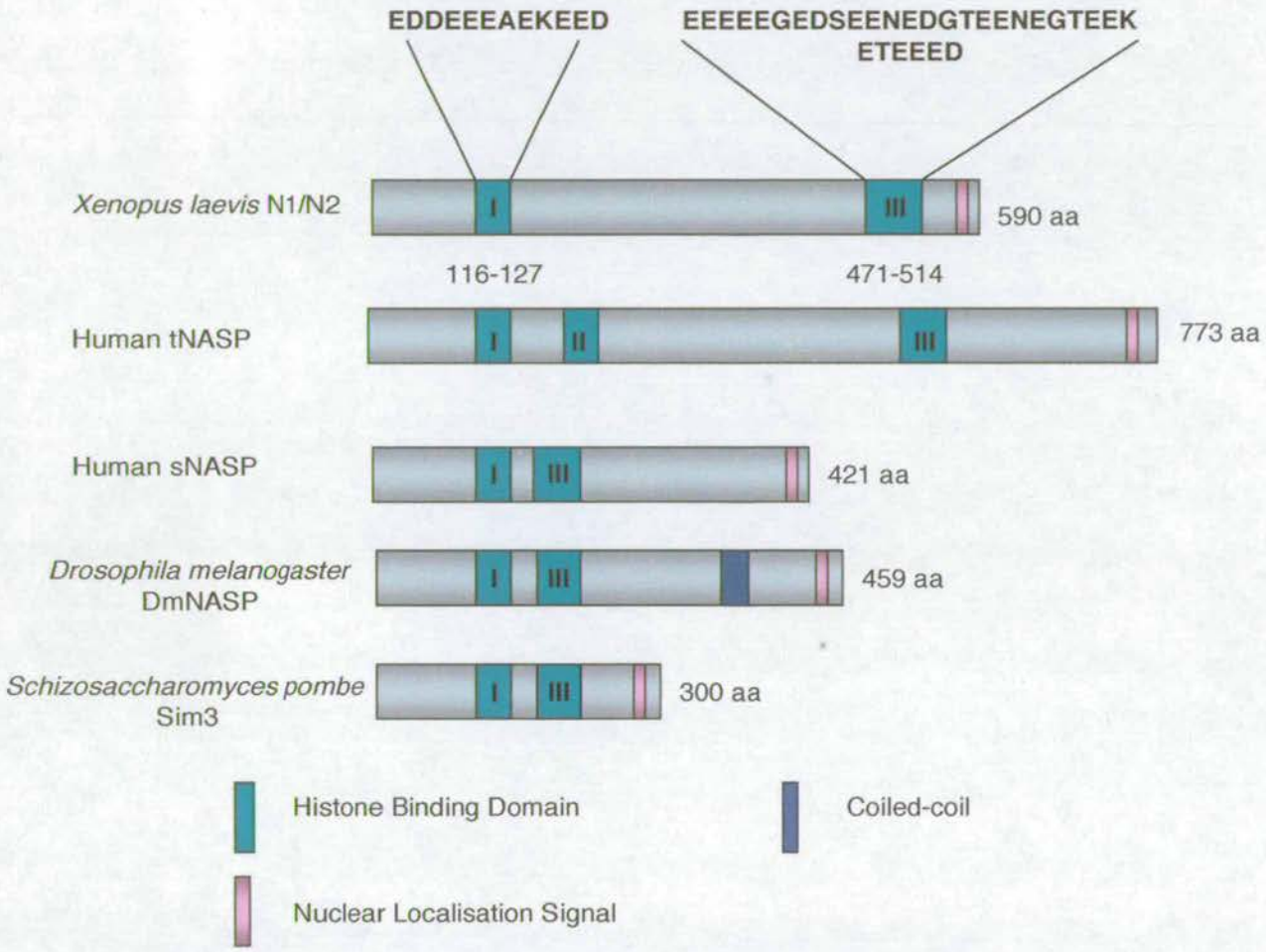


Figure 6-1. Conserved histone binding domains in NASP homologues.

Mammalian tNASP encodes a 773 amino acid protein with three histone binding domains. The 421 amino acid mammalian sNASP arises due to alternate splicing and lacks the second histone binding domain. Most of the third histone binding domain is lost in species outside *Xenopus laevis*. The first histone binding domain of *Xenopus laevis* is 70% identical and 90% similar to the human NASP domain. The third histone binding domain of *Xenopus laevis* is 48% identical and 71% similar to the corresponding domain in human NASP. *Schizosaccharomyces pombe* Sim3 shows approximately 24% identity to DmNASP (see chapter 4, Figure 4-4 for Clustal X alignment of Sim3/NASP homologues).

two rabbits (R19082, R19083) were injected with a 30 amino acid DmNASP N terminal peptide (1804) and two rabbits (R19080, R19081) were injected with a 30 amino acid DmNASP C terminal peptide (1803). No similar peptides were detected in the *Drosophila* genome. Pre-immune sera and final bleeds from the immunised rabbits were diluted (1 in 1000) and used for western blotting against GST-DmNASP fusion protein (Figure 6-2). Though there was a weak signal for pre-immune serum against GST-NASP (82 kDa), the signal for the final bleed was many times stronger. Neither pre-immune serum nor final bleeds recognised GST alone, suggesting that the antibody is specifically recognising DmNASP.

Sera from immunised rabbits were then affinity purified against the DmNASP N and C terminal peptides (1804 and 1803) in order to reduce non-specific background signals. Antibodies were eluted under acidic and basic conditions to optimise the recovery of active antibodies, with acidic conditions eluting the highest titre of antibodies. Eluted antibodies were then tested by western analysis against total protein extracts from wild type *D. melanogaster* S2 cells and were found to recognise a band for DmNASP at 70 kDa that migrates slower than expected (predicted molecular weight of DmNASP is 54 kDa), (Figure 6-3).

6.3 DmNASP is localised to the nucleus

To determine the localisation of DmNASP, S2 cells were fixed and stained with either pre-immune rabbit serum or affinity purified anti-DmNASP N or C terminal antibodies (Figure 6-4). Pre-immune sera showed faint staining throughout the cell (1803, C terminal) and variable punctate staining in both nucleus and cytoplasm (1804, N terminal). Affinity purified anti-DmNASP C and N terminal antibodies showed slightly different localisation patterns. Affinity purified anti-DmNASP N brightly stained the nucleus and appears to be localised to a rim on the periphery of the DAPI stained nucleus. Affinity purified anti-DmNASP C also stained the nucleus brightly and very little, if any, cytoplasmic staining was observed. These differences in localisation can be explained by the fact that N and C terminal anti-DmNASP antibodies recognise different regions of the DmNASP protein. Previously, it has been reported that human sNASP is cell cycle regulated and accumulates in S phase (Richardson et al., 2000), whereas both *Xenopus* N1/N2 (Kleinschmidt et al., 1988) and Sim3 (see chapter 4) localise to the nucleus throughout the cell cycle. Both N and C terminal DmNASP antibodies showed variation in the intensity of nuclear staining and some cells were negative for DmNASP staining. These results suggest that DmNASP may be cell cycle regulated in flies. However, as increasing the concentration of antibody used for staining resulted in an increased number of cells with nuclear staining, it is difficult to distinguish between cell cycle regulation of DmNASP or that simply a low titre of DmNASP antibodies results in more efficient staining of some cells and less efficient

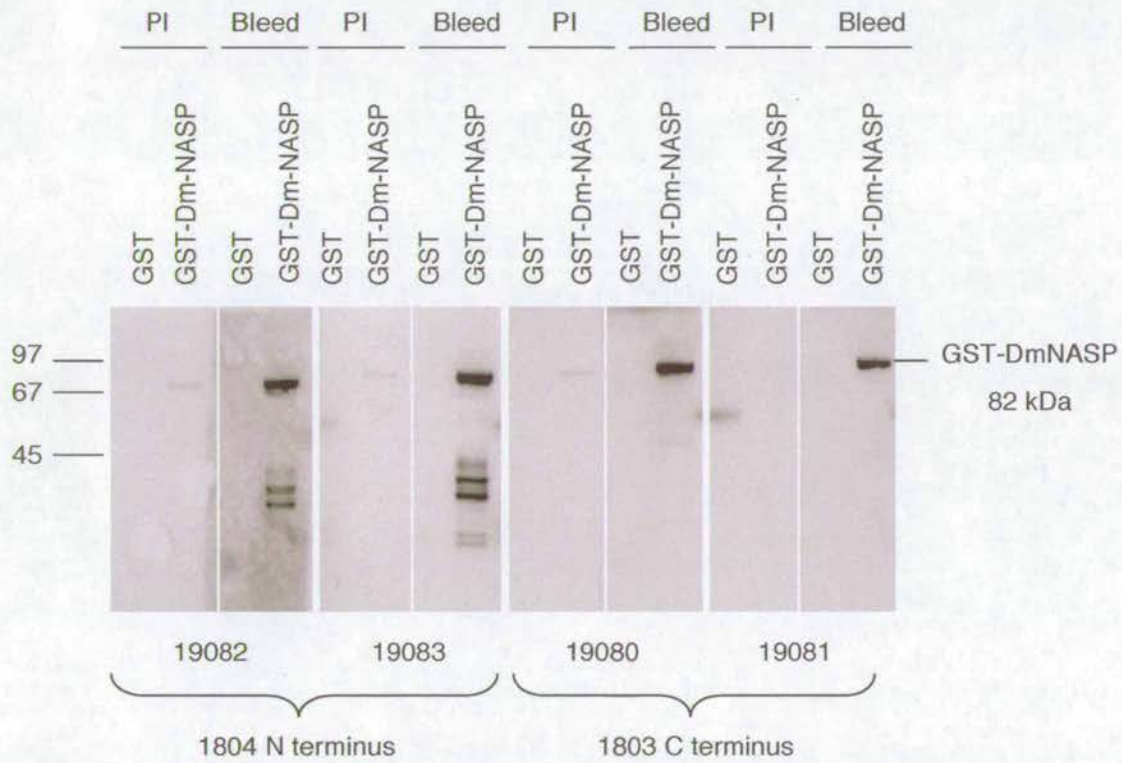


Figure 6-2. Anti-DmNASP serum can recognise GST-NASP fusion protein by western blot.

Rabbits 19082 and 19083 were immunised with a DmNASP C terminal peptide (1803). Rabbits 19080 and 19081 were immunised with DmNASP N terminal peptide (1804). Pre-immune serum (PI) and final bleeds (Bleed) from each rabbit were diluted 1:1000 in PBS-Tween and were used to probe a membrane with GST and GST-DmNASP immobilised. Though there was a weak signal for pre-immune sera against GST-DmNASP (82 kDa), the signal for the final bleed was many times stronger and did not recognise GST alone.

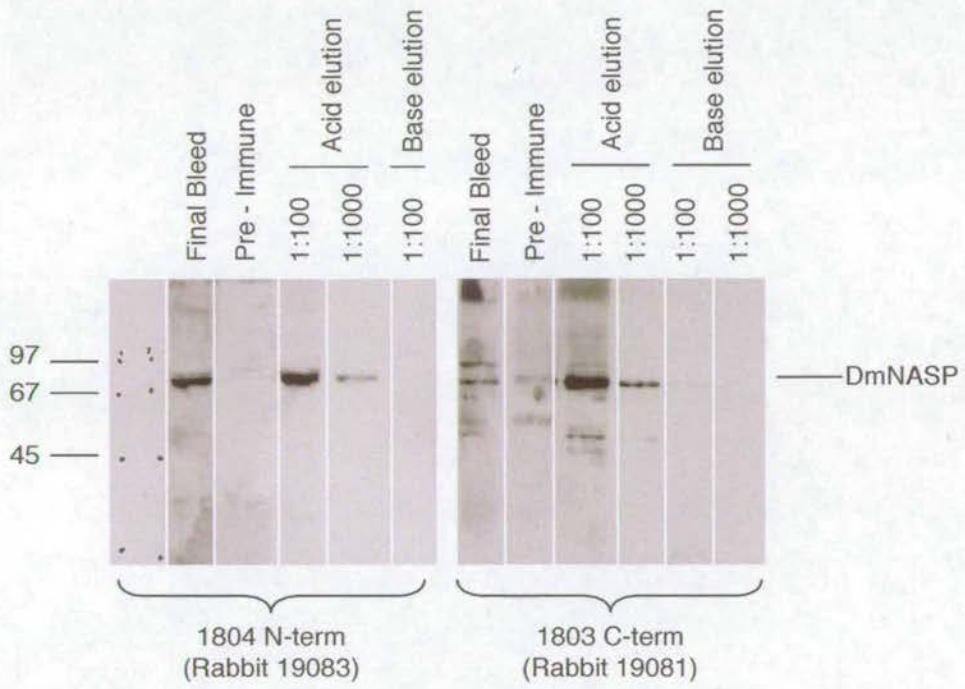


Figure 6-3. Affinity purified anti-DmNASP antibodies recognise a single band in *Drosophila melanogaster* S2 whole cell extracts.

N terminal and C terminal peptides were covalently linked to the SulphoLink coupling gel and were used for affinity purification of anti-DmNASP diluted serum. Anti-DmNASP antibodies were eluted under acidic and basic conditions and were used for western blotting against total protein extracts from S2 cells. Anti-DmNASP C and N terminal antibodies eluted under acidic conditions gave a stronger signal and recognised a band representing DmNASP running at about 70 kDa (predicted molecular weight of DmNASP is 54 kDa). Pre-immune serum and final bleed from the rabbit were also tested.

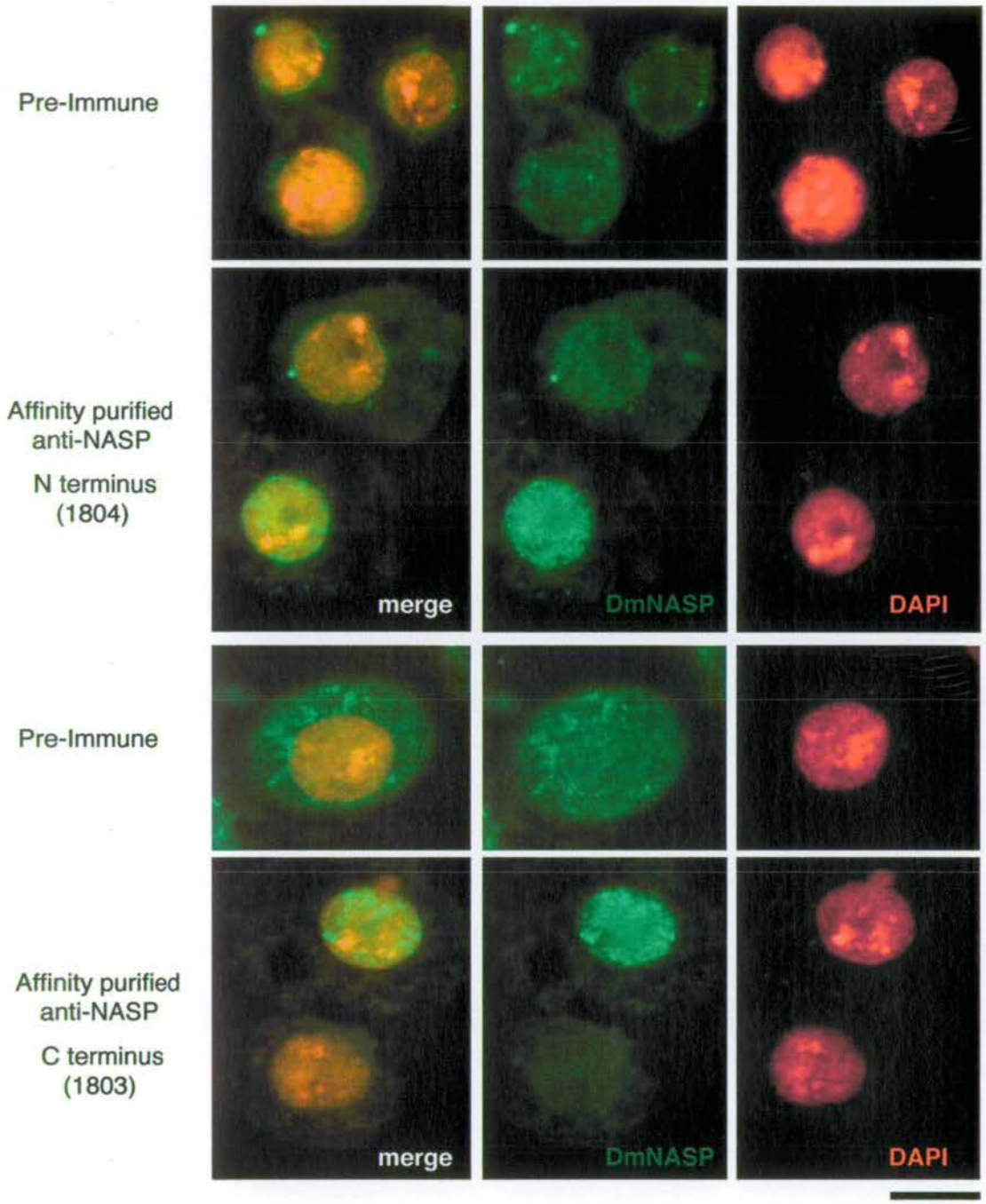


Figure 6-4. Localisation of DmNASP using affinity purified anti-DmNASP antibodies.

Pre-immune serum and anti-DmNASP N terminal (peptide 1804, rabbit 19082) or anti-DmNASP C terminal (peptide 1803, rabbit 19080) were used to localise DmNASP in S2 cells (1:100 dilution of each). S2 cells were fixed in 4% PFA and were stained with anti-DmNASP antibodies (green) and DNA was stained with DAPI (red) and were viewed by fluorescent microscopy. Both N and C terminal anti-DmNASP antibodies show a nuclear localisation pattern, which was not observed with cells stained with pre-immune serum. Bar 5 μm.

staining of others. This could be further tested by assaying levels of DmNASP RNA and protein in S2 cells blocked at different stages of the cell cycle.

6.4 Knockdown of DmNASP by RNAi in S2 cells does not affect the localisation of endogenous CENP-A^{CID}

As described in chapter 5, Sim3 is required for the association of endogenous CENP-A^{Cnp1} with the centromere in fission yeast. To determine whether DmNASP plays a role in the localisation of CENP-A^{CID} to the centromere in *Drosophila*, RNAi of DmNASP was carried out in S2 cells. DmNASP dsRNA was synthesized *in vitro* and 15 μg of dsRNA added to S2 cells in culture at hour 0 and again at hour 72 to maintain the knockdown. EB1 dsRNA was also prepared and EB1 knockdown was carried out according to the previously published protocol (Rogers et al., 2002). EB1 is a microtubule plus end binding protein that plays a critical role in the assembly, dynamics and positioning of the mitotic spindle (Rogers et al., 2002). Thus, EB1 knockdown served as a positive control that the RNAi procedure was working and also as a negative control, as EB1 is not expected to affect the localisation of CENP-A^{CID}. A mock treated 'no RNA' control was also included in each experiment.

After the addition of the DmNASP dsRNA, total protein was extracted from cells every 24 hours for 6 days and the knockdown of DmNASP protein was monitored by western blotting using affinity purified anti-DmNASP (N terminal antibody was used for all western analysis), (Figure 6-5A). After 24 hours treatment with 15 μg dsRNA, DmNASP protein levels were diminished and remained depleted throughout the experiment up to the last time point of 144 hours. Anti-EB1 was used as a loading control and was unaffected by the knockdown of DmNASP. After 72 hours treatment with 15 μg EB1 dsRNA, EB1 protein levels were reduced and EB1 was efficiently knocked down after 144 treatment with EB1 dsRNA (Figure 6-5B). In order to estimate the extent of the DmNASP knockdown, extracts from the mock treated control and DmNASP RNAi were diluted, loaded on an SDS-PAGE gel and probed with anti-DmNASP and anti-EB1 antibodies. By dilution of an extract from the control sample, it was revealed that levels of DmNASP protein were estimated to be reduced to less than 10% of normal levels (Figure 6-6).

In addition to protein extraction, cells were fixed and processed for anti-CENP-A^{CID} (gift from F. Azorin) immuno-fluorescence microscopy and DNA was stained with DAPI at each time point. After 24 hours, when levels of DmNASP protein were already reduced, the localisation of CENP-A^{CID} in DmNASP dsRNA treated cells was identical to EB1 dsRNA treated cells and mock treated control (Figure 6-7). No change in CENP-A^{CID} localisation was observed throughout the 6-day period, as shown by anti-CENP-A^{CID} and DAPI staining after 144 hours treatment with DmNASP dsRNA (Figure 6-8). Depletion of CENP-A^{CID} by RNAi in *Drosophila* Kc tissue-culture cells has been shown to result in aberrant chromosome

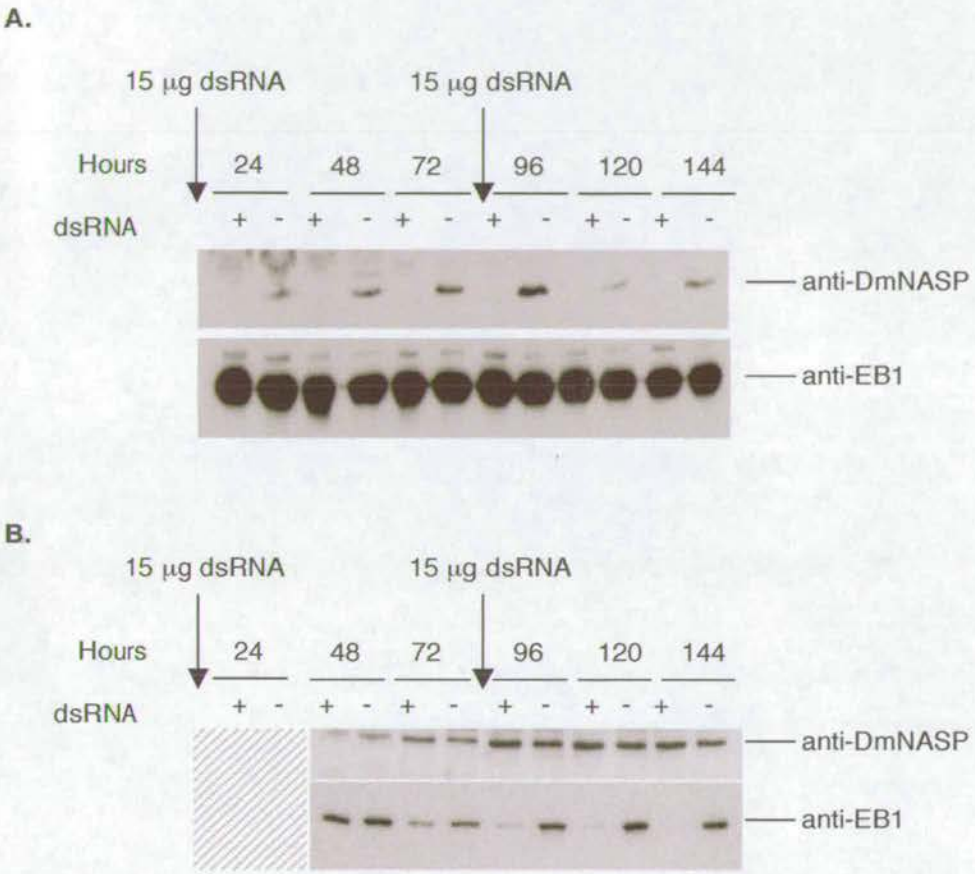


Figure 6-5. Knockdown of DmNASP and EB1 by RNAi in S2 cells.

A. Monitoring DmNASP protein levels upon the addition of 15 μg DmNASP dsRNA to S2 cells at hour 0 and again at hour 72 by anti-DmNASP western blot shows that knockdown is achieved after 24 hours and is maintained until 144 hours. EB1 levels were used as a loading control and remained unaltered throughout. 5 x 10^4 cells are loaded per lane.

B. Monitoring EB1 protein levels upon the addition of 15 μg EB1 dsRNA to S2 cells at hour 0 and again at hour 72 by anti-EB1 western blot shows that knockdown is achieved after 144 hours. NASP levels are used as a loading control were unaffected when EB1 levels declined due to knockdown. The 24 hour time point is not shown due to contamination of sample. 5 x 10^4 cells are loaded per lane.

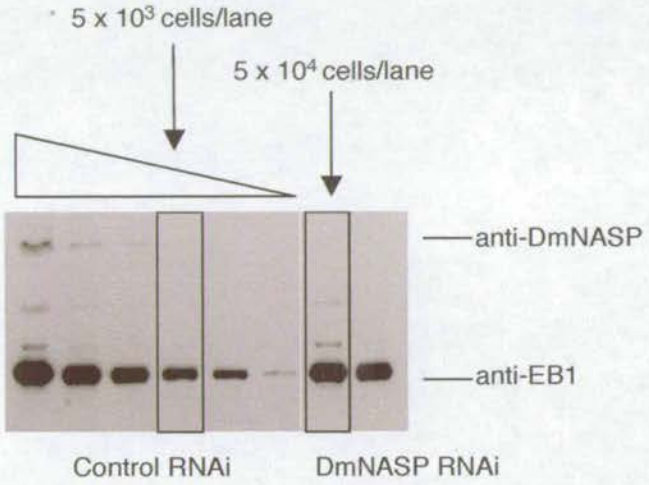


Figure 6-6. DmNASP is reduced to less than 10% of wild type levels by RNAi in S2 cells.

To estimate efficiency of the DmNASP knockdown, protein extracts from control (no RNA) and DmNASP RNAi samples were diluted 1:2 and were probed with anti-DmNASP and anti-EB1 by western blot. Protein from 5 x 10^4 RNAi treated cells was approximately equivalent to protein from 5 x 10^3 control cells. This suggests that in DmNASP dsRNA treated cells the level of DmNASP protein is reduced to <10% of normal levels.

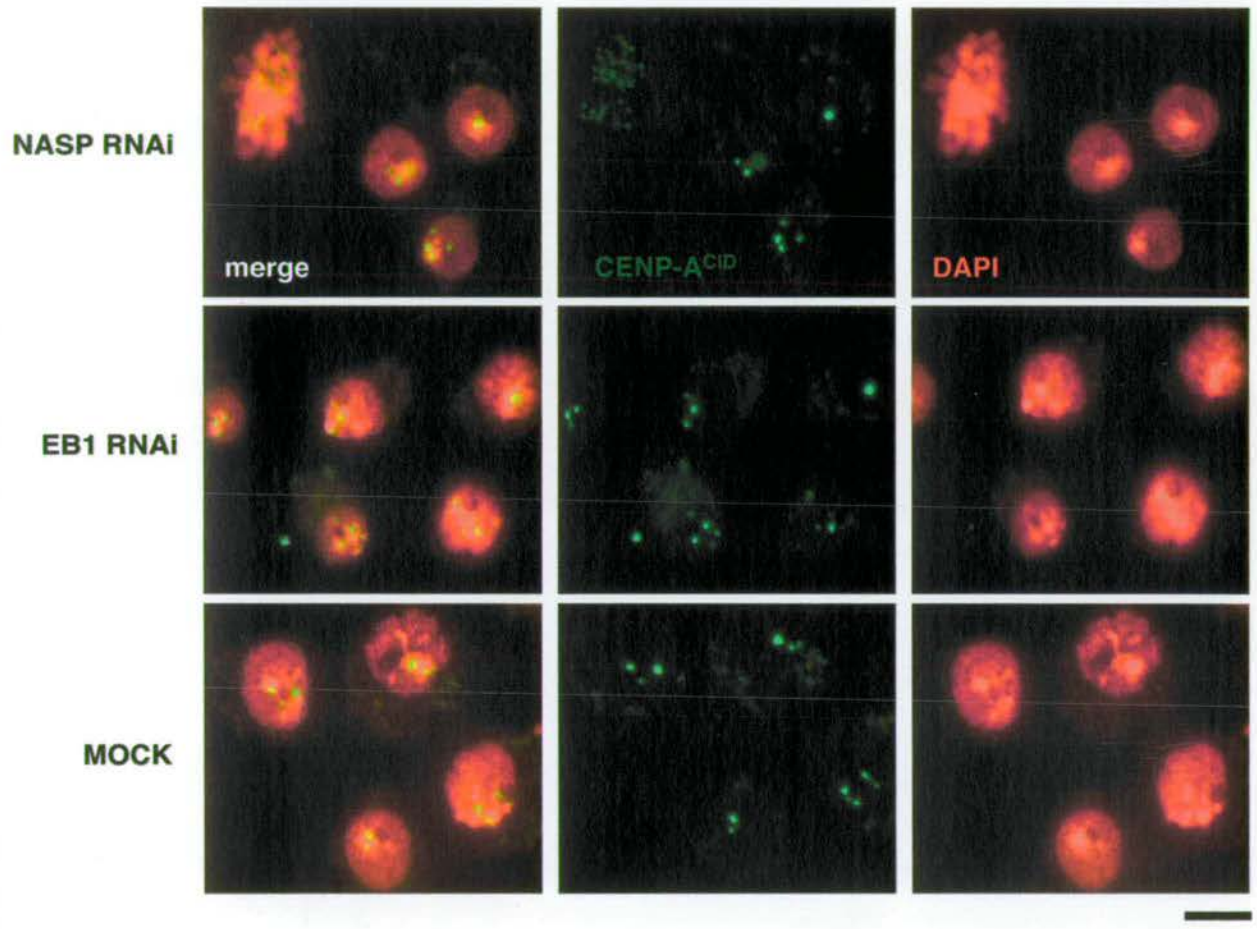


Figure 6-7. CENP-A^{CID} remains localised in cells treated with DmNASP dsRNA (24 hours).

Cells were fixed and stained with anti-CENP-A^{CID} (green) and DNA was stained with DAPI (red) after 24 hours treatment with 15 μg DmNASP dsRNA. CENP-A^{CID} remains localised in cells with depleted DmNASP, with depleted EB1 and mock treated control. Bar 5 μm.

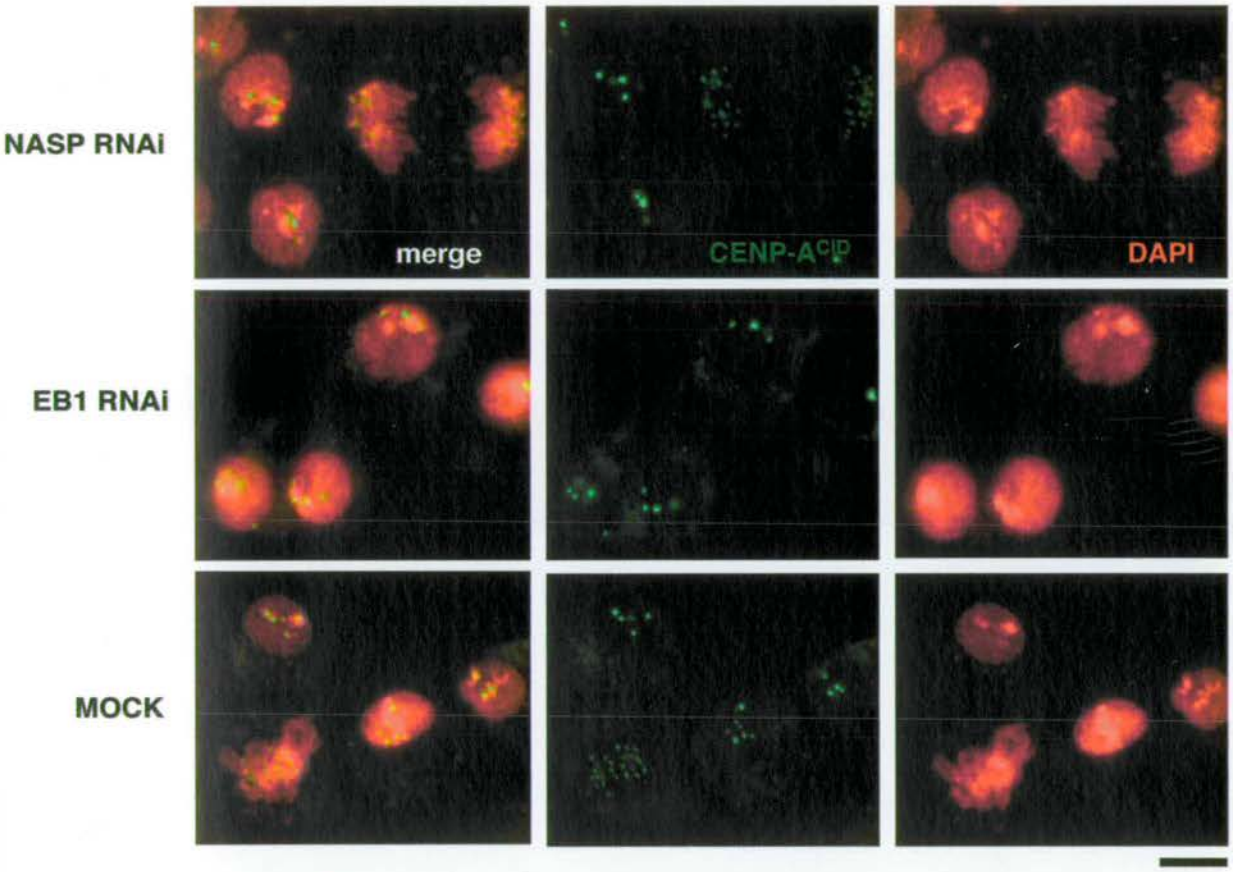


Figure 6-8. CENP-A^{CID} remains localised in cells treated with DmNASP dsRNA (144 hours).

Cells were fixed and stained with anti-CENP-A^{CID} (green) and DNA was stained with DAPI (red) after 144 hours treatment with 15 μg DmNASP dsRNA. CENP-A^{CID} remains localised in cells with depleted DmNASP, with depleted EB1 and mock treated control. Bar 5 μm.

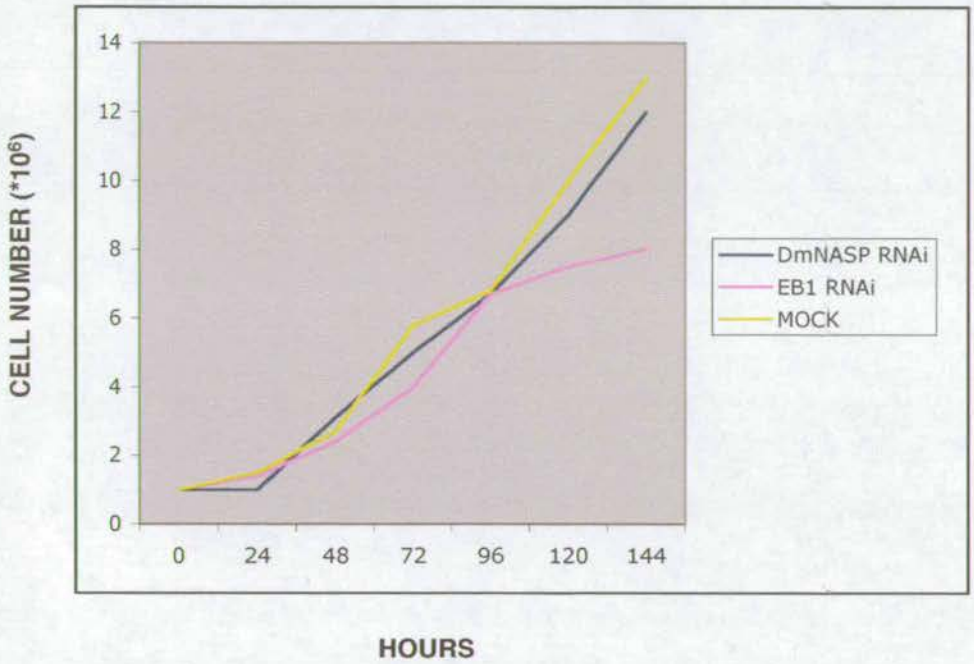


Figure 6-9. S2 cell growth after DmNASP dsRNA treatment.

S2 cells were treated with 15μg DmNASP dsRNA, EB1 dsRNA or mock treated control at 0 hours and after 72 hours. Total number of cells were counted (x 10^6) every 24 hours and are plotted on the graph against time (Blue = DmNASP RNAi, Pink = EB1 RNAi, Yellow = Mock RNAi).

segregation in mitosis (Blower and Karpen, 2001). However, mitotic defects were never observed in DmNASP RNAi treated or untreated cells in my experiments. Cell growth was unaffected by the knockdown as DmNASP RNAi cells grew with wild type dynamics (Figure 6-9). The knockdown of EB1 resulted in a slower cell growth rate than either control or DmNASP RNAi cells. This is consistent with published results where depletion of EB1 by RNAi in S2 cells resulted in a delay in cell cycle progression (Rogers et al., 2002).

6.5 Knockdown of DmNASP by RNAi in S2 cells expressing CENP-A^{CID}-GFP does not affect the localisation of CENP-A^{CID}-GFP

Knockdown of DmNASP by RNAi in S2 cells did not result in any change in CENP-A^{CID} localisation by anti-CENP-A^{CID} immunostaining. RNAi of DmNASP was next carried out in S2 cells that were expressing CENP-A^{CID}-GFP, which may allow changes in CENP-A^{CID}-GFP localisation to be detected more readily and immunostaining is not required to visualise CENP-A^{CID}-GFP. A stable S2 cell line expressing CENP-A^{CID}-GFP fusion protein under the control of the inducible metallothionein promoter (as described in Heun et al., 2006) was obtained from the Karpen laboratory, University of California, Berkeley. These cells were selected with 100 μg/ml hygromycin and uninduced cells constitutively express CENP-A^{CID}-GFP at a low level, estimated to be approximately 2 times the endogenous level of CENP-A^{CID} (Barbara Mellone, personal communication). As described above, DmNASP dsRNA was synthesized *in vitro* and 15 μg dsRNA added to S2 cells in culture at hour 0 and at hour 72. At each time point, cells were again processed for western blotting and fixed for fluorescent imaging of CENP-A^{CID}-GFP signal. Western blotting with anti-NASP antibody shows that protein was reduced after 24 hours treatment and was almost completely diminished after 144 hours treatment (Figure 6-10A). After 144 hours treatment with EB1 dsRNA, western blotting with anti-EB1 shows that EB1 protein levels are reduced (Figure 6-10B). As cells were constitutively expressing CENP-A^{CID}-GFP, total protein extracts were also probed with anti-GFP that was used as a loading control. It was also noted that levels of CENP-A^{CID}-GFP appeared to be stable and comparable in the DmNASP RNAi treated and untreated cells (Figure 6-10A).

After 24 hours, when levels of DmNASP protein were already reduced, the localisation of CENP-A^{CID}-GFP in DmNASP dsRNA treated cells was identical to pattern observed for EB1 dsRNA treated cells and also to mock treated control (Figure 6-11). No change in CENP-A^{CID}-GFP localisation was observed throughout the 6-day period, as shown by CENP-A^{CID}-GFP signal and DAPI staining after 144 hours treatment with DmNASP dsRNA (Figure 6-12). In addition, intensity of CENP-A^{CID}-GFP signal did not appear to be reduced in DmNASP RNAi treated cells compared to controls. As cells have different levels of CENP-A^{CID}-GFP expression to begin with, due to variable expression levels from the metallothionein promoter, it was not possible to quantify the intensity of the CENP-A^{CID}-

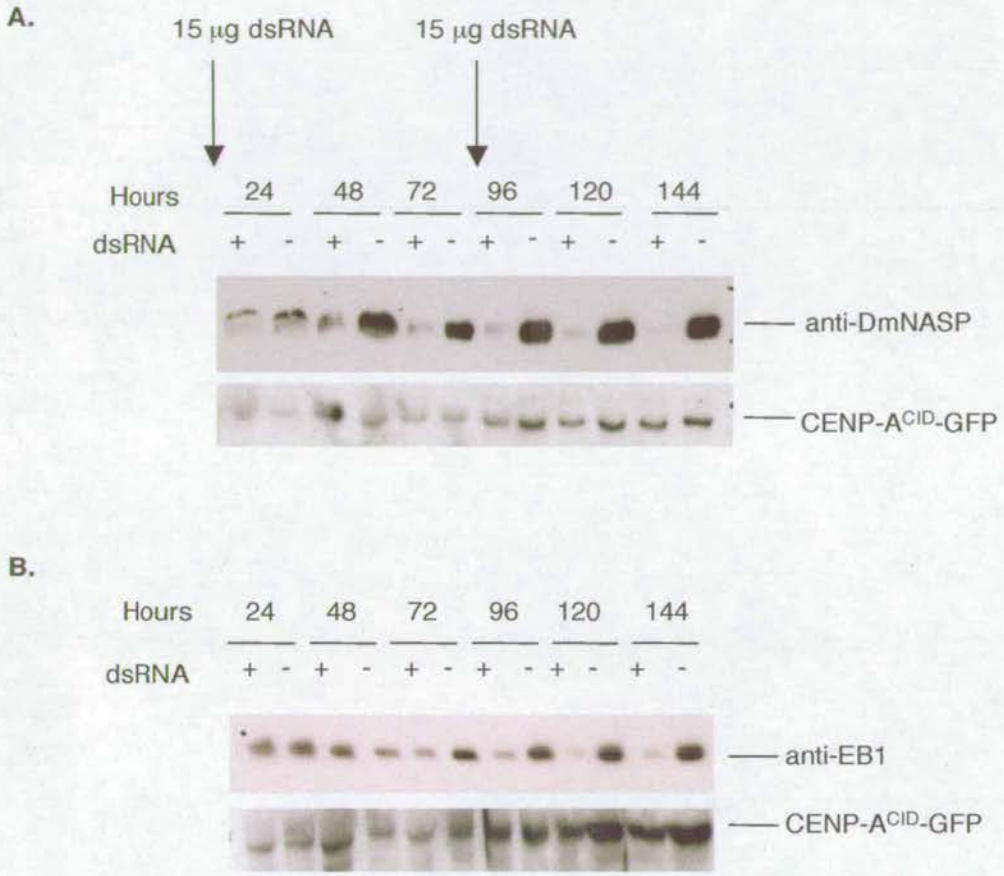


Figure 6-10. Knockdown of DmNASP and EB1 by RNAi in S2 cells expressing CENP-A^{CID}-GFP.

A Monitoring of DmNASP protein levels upon the addition of 15 μg dsRNA to S2 cells expressing CENP-A^{CID}-GFP at hour 0 and again at hour 72 by anti-DmNASP western blot. DmNASP protein is reduced after 24 hours and is efficiently depleted after 144 hours. Anti-GFP was used to detect CENP-A^{CID}-GFP. Levels of CENP-A^{CID}-GFP are stable in DmNASP dsRNA treated and untreated cells. 5 x 10^4 cells are loaded per lane.

B. Monitoring of EB1 protein levels upon the addition of 15 μg dsRNA to S2 cells expressing CENP-A^{CID}-GFP at hour 0 and again at hour 72 by anti-EB1 western blot. EB1 protein is efficiently depleted after 144 hours. Anti-GFP was used to detect CENP-A^{CID}-GFP. Levels of CENP-A^{CID}-GFP are stable in EB1 dsRNA treated and untreated cells as detected by anti-GFP western blot. 5 x 10^4 cells are loaded per lane.

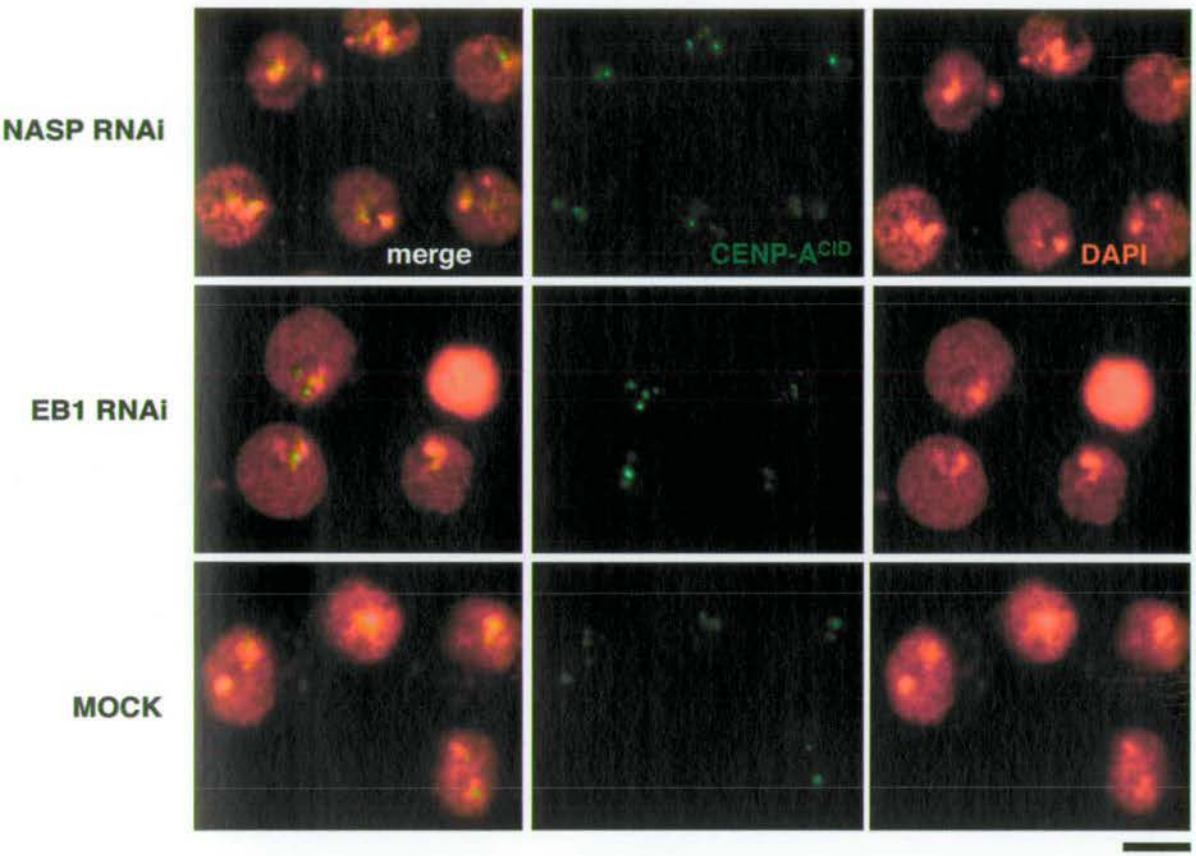


Figure 6-11. CENP-A^{CID}-GFP remains localised in cells treated with DmNASP dsRNA (24 hours).

Cells were fixed, DNA was stained with DAPI (red) and CENP-A^{CID}-GFP (green) was visualised by fluorescence microscopy after 24 hours treatment with 15 μg DmNASP dsRNA. CENP-A^{CID}-GFP remains localised in cells with depleted NASP, with depleted EB1 and mock treated controls. Bar 5 μm.

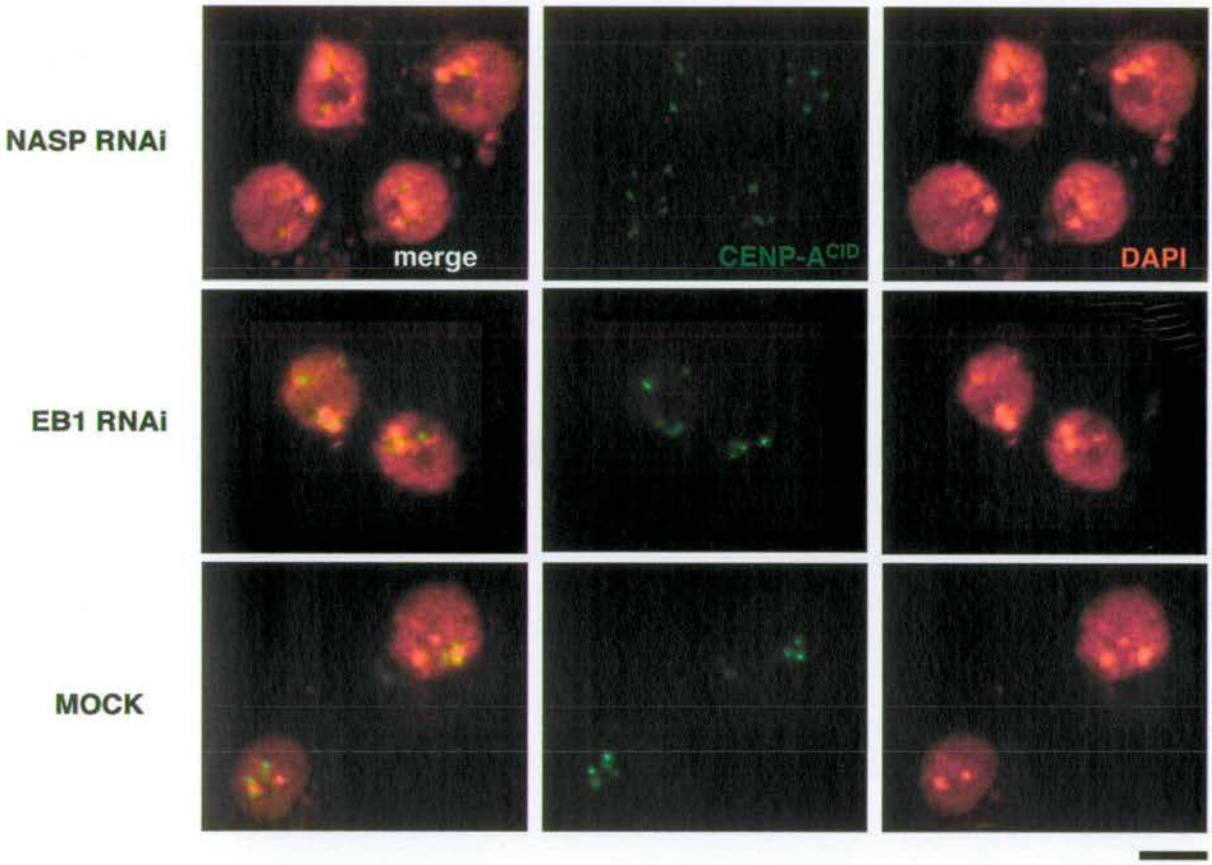


Figure 6-12. CENP-A^{CID}-GFP remains localised in cells treated with DmNASP dsRNA (144 hours).

Cells were fixed, DNA was stained with DAPI (red) and CENP-A^{CID}-GFP (green) was visualised by fluorescence microscopy after 144 hours treatment with 15 μg dsRNA. CENP-A^{CID}-GFP remains localised in cells with depleted NASP, with depleted EB1 and mock treated controls. Bar 5 μm.

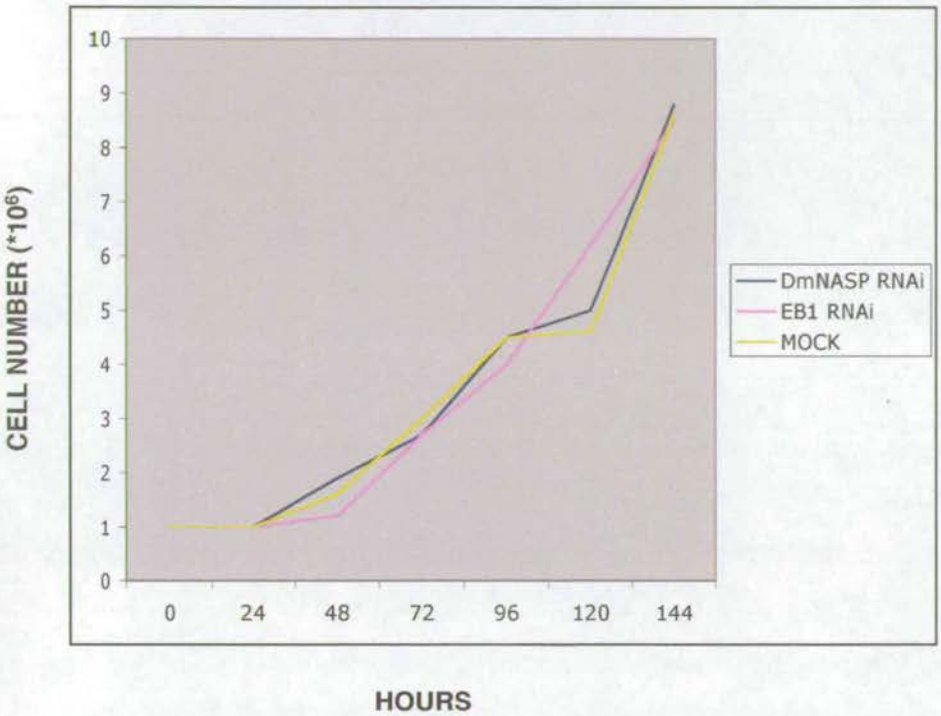


Figure 6-13. Growth of RNAi treated S2 cells expressing GFP-CENP-A^{CID} after DmNASP dsRNA treatment.

S2 cells were treated with 15μg DmNASP dsRNA, EB1 dsRNA or mock treated control at 0 hours and after 72 hours. Total number of cells were counted (x 10^6) every 24 hours and are plotted on the graph against time (Blue = DmNASP RNAi, Pink = EB1 RNAi, Yellow = Mock RNAi).

GFP fluorescent signal in a systematic manner. As observed in the previous DmNASP knockdown experiment in S2 cells, cell growth was unaffected by the knockdown as DmNASP RNAi in S2 cells over-expressing CENP-A^{CID}-GFP and cells grew with wild type dynamics (Figure 6-13). Unexpectedly, for this experiment, a slowing of growth rate was not observed in cells treated with EB1 dsRNA (as was observed by Rogers et al., 2002 and Figure 6-9). This may be due to the fact that the knockdown of EB1 may not have been as efficient in this experiment and some residual EB1 protein remains after 144 hours treatment with EB1 dsRNA (Figure 6-10B). Thus, it appears from both RNAi experiments described above that DmNASP does not affect the localisation of endogenous CENP-A^{CID} nor is it required for the localisation of constitutively over-expressed CENP-A^{CID}-GFP.

6.6 Production of GST-fusion proteins and *in vitro* binding studies with *Drosophila* DmNASP/histones.

To investigate whether DmNASP has the ability to interact with histones, genes encoding *D. melanogaster* GST fusion proteins (GST-DmNASP, GST-CENP-A^{CID}, GST-histone H3.3, GST-histone H4 and GST-histone H1) were cloned, sequenced and expressed in *E. coli* (Figure 6-14). Not all fusion proteins expressed as well as each other, with GST-DmNASP showing the highest level of expression. GST-fusion proteins were then utilised in pull-down experiments with labelled ^{35}S DmNASP and ^{35}S CENP-A^{CID}, which were produced by *in vitro* transcription and translation (Figure 6-15A and 6-15B). ^{35}S CENP-A^{CID} did not bind to GST-DmNASP, and reciprocally, ^{35}S DmNASP was unable to bind to GST-CENP-A^{CID} under these conditions. ^{35}S CENP-A^{CID} was found to interact with GST-CENP-A^{CID}, suggesting that CENP-A^{CID} can form a homodimer. As expected, ^{35}S CENP-A^{CID} was also found to interact with the GST-histone H4, suggesting that CENP-A^{CID} and GST-H4 can form a heteromer *in vitro*. In addition, CENP-A^{CID} was found to interact with GST-histone H1. The interaction between the CENP-A histone variant and the linker histone H1 has not previously been reported.

^{35}S DmNASP was found to interact weakly, but consistently, with histone H3.3 under the pulldown conditions (Figure 6-15B). This correlates with the finding that in HeLa cells human sNASP and tNASP co-purify with the isolated HIRA-H3.3 assembly complex (Tagami et al., 2004). In an attempt to confirm the interaction between DmNASP and H3.3 *in vivo*, immunoprecipitation with anti-DmNASP N terminal and C terminal antibodies was carried out in extracts from wild type S2 cells. Using an antibody that recognises both canonical H3 and the H3.3 variant, H3 was visible in whole cell extracts, but it was not visible in the anti-DmNASP immunoprecipitated fractions (not shown). The failure to detect an interaction between DmNASP and H3 may be due to the differences in composition of the buffers used for the *in vitro* interaction (PBS + 1% Triton X-100) and for co-immunoprecipitation interactions *in vivo* (RIPA buffer = 150 mM NaCl, 1% NP-40, 0.5%

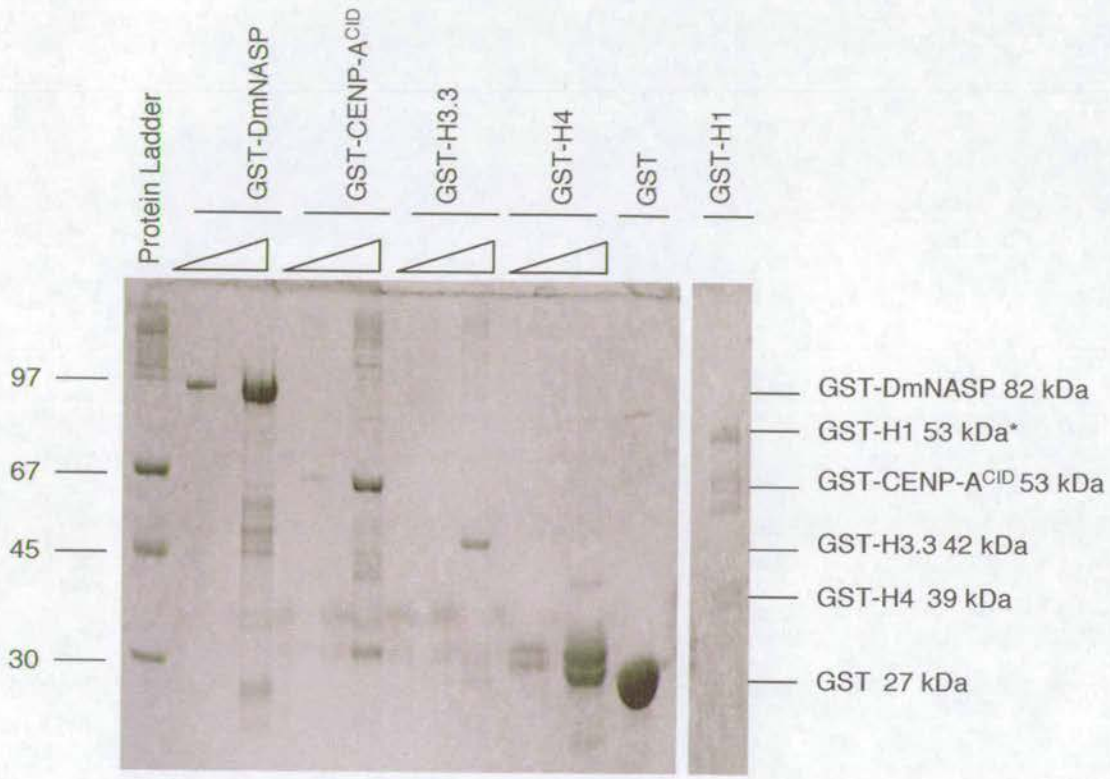


Figure 6-14. Expression of *D. melanogaster* GST fusion proteins in *E.coli.*

1 μl and 10 μl of each fusion protein was run on an SDS-PAGE gel and Coomassie stained. Molecular weights of the fusion proteins are indicated. *The predicted molecular weight of GST-H1 is 53 kDa, however it was found to migrate slower than expected on the SDS-PAGE gel.

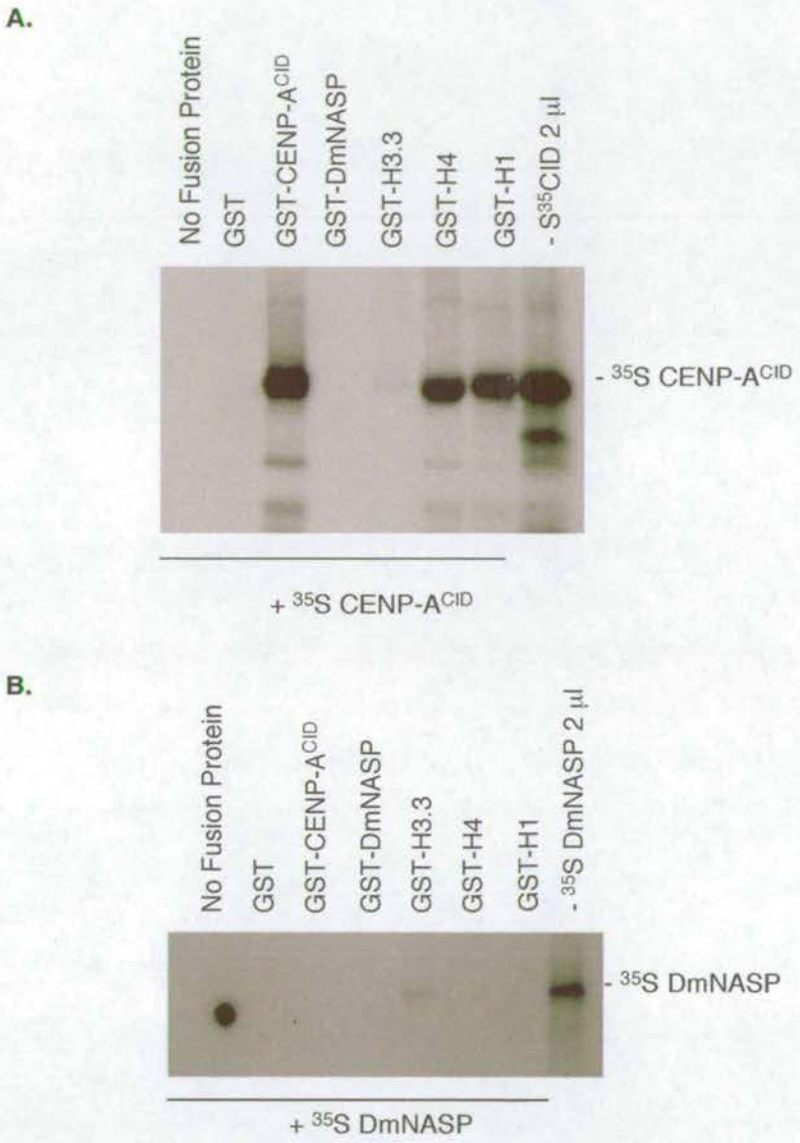


Figure 6-15. Binding of CENP-A^{CID} and DmNASP to histones *in vitro*.

^{35}S-labelled DmNASP and ^{35}S-labelled CENP-A^{CID} were produced by *in vitro* transcription and translation and tested for binding to GST-DmNASP, GST- CENP-A^{CID}, GST-H3.3 and GST-H4. Equal amounts of GST fusion protein (4 µg) and 4 µl of ^{35}S-labelled protein was added to each reaction. Reactions were carried out in PBS + 0.1% Triton-X.

A. ^{35}S CENP-A^{CID} can bind GST-CENP-A^{CID}, GST-H4 and GST-H1.

B. ^{35}S DmNASP can weakly bind GST-H3.3.

Deoxycholate, 0.1% SDS, 50 mM Tris pH 8, 10 mM NaF, 0.4 mM EDTA, 10% glycerol). Indeed, when the *in vitro* pulldown was carried out in the RIPA buffer, the interaction between ^{35}S DmNASP and GST-H3.3 was lost (not shown).

DISCUSSION

This chapter describes a characterisation of the *Drosophila melanogaster* Sim3/NASP homologue, designated DmNASP. The *Drosophila* tissue culture S2 cell line was utilised to determine the localisation of DmNASP. S2 cells were stained with affinity purified anti-DmNASP N and C terminal antibodies and like Sim3, DmNASP was found to localise to the nucleus and it is possible that DmNASP is cell cycle regulated. *Drosophila melanogaster* S2 cells are easily amenable to the knockdown of a gene of interest using double stranded RNA-mediated (RNAi). In this study, DmNASP was depleted and its roles in chromosome segregation in mitosis and any effects on the localisation of CENP-A^{CID} were monitored.

Drosophila melanogaster DmNASP is not required for the localisation of endogenous CENP-A^{CID} to centromeres

Knockdown of DmNASP did not result in any change in the localisation of endogenous CENP-A^{CID}, which was examined by anti-CENP-A^{CID} antibody staining. This result was confirmed in a cell line where CENP-A^{CID}-GFP is expressed under the inducible metallothionein promoter (uninduced cells were estimated express CENP-A^{CID}-GFP at twice the endogenous level of CENP-A^{CID}, B. Mellone, personal communication). Here, knockdown of DmNASP did not affect the localisation of CENP-A^{CID}-GFP, which was examined by fluorescence microscopy. Hence, unlike Sim3, depletion of DmNASP does not appear to affect the localisation of pre-existing CENP-A^{CID} at centromeres. Also, DmNASP depletion to less than 10% of wild type levels by RNAi did not give rise to any chromosome segregation defects, suggesting that, unlike *sim3* mutants, mitosis was unaffected in these cells.

The experiments using the CENP-A^{CID}-GFP cell line was also carried out under conditions where the metallothionein promoter was induced by the addition of 250 μM of copper sulphate ($CuSO_4$) to the medium as described in Heun et al. (2006), (carried out in collaboration with Barbara Mellone, Karpen laboratory, UC, Berkeley). This induction results in an increased expression of new CENP-A^{CID}-GFP approximately 30 fold higher than endogenous levels of CENP-A^{CID}. Due to leakage of the metallothionein promoter, this cell line expresses a basal level of CENP-A^{CID}-GFP that are localised as spots at centromere. For this reason, it was not possible to examine the effect of DmNASP depletion on the incorporation of new CENP-A^{CID}-GFP at centromeres using this system. It was possible however to determine, whether new CENP-A^{CID}-GFP becomes associated ectopically with

other chromatin sites or whether the import of CENP-A^{CID}-GFP is affected on depletion of DmNASP. However, under these conditions, cells that were depleted of DmNASP did not show any defects in import of CENP-A^{CID}-GFP into the nucleus and CENP-A^{CID}-GFP remained localised to centromeres as previously observed.

It has been shown that the replication of human CENP-A chromatin is a semi-conservative process (Shelby et al., 2000). In this way, parental CENP-A is equally partitioned to daughter cells and is reduced by 50% with each round of replication. As 50% of the CENP-A^{CID} that is already at the centromere remains there in every cell cycle, it may be difficult to detect a reduction in CENP-A^{CID} association with centromeres on depletion of DmNASP. Indeed, a recent study by Okada et al. (2006) has shown that the CENP-H-I complex contributes to the nascent CENP-A incorporation into centromeres in HeLa cells (Okada et al., 2006). However, a reduction in the level of endogenous CENP-A at kinetochores in the mutant cells was not detected (Okada et al., 2006). As demonstrated in chapter 5, Sim3 is required for the localisation of newly synthesized CENP-A^{Cnp1} to centromeres. Therefore, it would be interesting to investigate whether DmNASP is required for the localisation of CENP-A^{CID} that is absolutely new to fly centromeres. To address this question, however, a more suitable inducible promoter than the metallothionein promoter, that does not exhibit leaky expression, is required.

DmNASP may act as a histone H3.3 chaperone in *Drosophila melanogaster*

As mammalian NASP and *Xenopus* N1/N2 have been shown to bind histones, the ability of DmNASP to bind to histones was investigated by *in vitro* pull down assays using GST-fusion proteins. ^{35}S DmNASP, did not bind to GST-CENP-A^{CID}, but was found to interact weakly with histone H3.3 under these conditions. As human NASP has been shown to co-purify with the H3.3-HIRA assembly complex (Tagami et al., 2004), it is possible that in flies, DmNASP plays a role in the assembly of histone H3.3 in a replication independent pathway and is not specialised for assembly of the histone H3 variant CENP-A^{CID} as it is in fission yeast.

Under these assay conditions, ^{35}S CENP-A^{CID} was found to form a homodimer with itself, which correlates with the fact that CENP-A^{CID} nucleosomes are thought to be homotypic and most likely consist of two copies of CENP-A^{CID} (Blower et al., 2002). This finding is also consistent with studies on the stoichiometry of CENP-A molecules found in the CENP-A nucleosome in human cells (Shelby et al., 1997, Yoda et al., 2000). In addition, ^{35}S CENP-A^{CID} was found to interact with GST-H4, which is expected, as CENP-A is known to replace H3 in the nucleosome and forms dimers with H4 (Shelby et al., 1997, Yoda et al., 2000, Blower et al., 2002). ^{35}S CENP-A^{CID} was also found to interact with histone H1. It is

tempting to speculate that interactions between CENP-A^{CID} and the linker histone H1 may contribute to the formation of the specialised chromatin at centromeres.

Possible roles of NASP in higher eukaryotes

Although the DmNASP does not appear to be required for the localisation of endogenous CENP-A^{CID} at centromeres, it is possible that DmNASP plays a role in the association of newly synthesized CENP-A^{CID} to centromeres and further investigation is worthwhile. It is also possible that in flies, other proteins or pathways, which facilitate the assembly of CENP-A^{CID} at centromeres, compensate the loss of functional DmNASP. Redundancy may also explain why no obvious phenotypes, such as chromosome segregation defects or CENP-A^{CID} association with centromeres, were observed on DmNASP depletion by RNAi. In addition, although depletion of DmNASP by RNAi was achieved with approximately 90% or more efficiency, the residual 10% or less of DmNASP protein remaining in the cell may be sufficient for DmNASP to carry out its normal cellular functions. For this reason, it would be of interest to carry out double knockdowns of DmNASP and other factors implicated in CENP-A^{CID} chromatin assembly. One such candidate is *Drosophila* p55 (RpAp48 in humans) that has recently been shown to facilitate the assembly of CENP-A^{CID} nucleosomes *in vitro* (Furuyama et al., 2006). RpAp48 is a common subunit of the H3.1 and H3.3-specific nucleosome assembly complexes isolated from HeLa cells (Tagami et al., 2004). In addition, the fission yeast p55 homologue, Mis16, is required for association of with centromeres and double depletion of Mis6 homologues RbAp46 and RbAp48 in HeLa cells results is delocalised CENP-A from centromeres (Hayashi et al., 2004). Another possibility is that NASP contributes to CENP-A chromatin assembly at centromeres in other higher eukaryotes, which studies to date have not addressed.

NASP has also been found to be a binding partner of chaperone heat shock protein 90 (HSP90) and the DNA repair proteins Ku70/Ku80 and DNA-activated protein kinase (DNA-PK) in HeLa cells (Alekseev et al., 2005). As both Ku70/Ku80 and DNA-PK are necessary for double strand break repair by non-homologous end joining, this indicates that in higher eukaryotes NASP may play an additional role in DNA repair events. Also, tNASP has been isolated in a complex with histone H1 in HeLa cells (Alekseev et al., 2003). As tNASP-H1 complexes can transfer H1 to DNA and tNASP and H1 have identical mobilities within the nucleus, this supports an additional role for tNASP as a linker histone chaperone (Alekseev et al., 2003).

Both tNASP and sNASP are common components of the DNA replication-dependent CAF1 and DNA replication-independent HIRA pathways (Tagami et al., 2004). NASP may function in these complexes as a histone donor providing a ready supply of histones for deposition at chromatin assembly. Indeed, human NASP has also been isolated in a multi

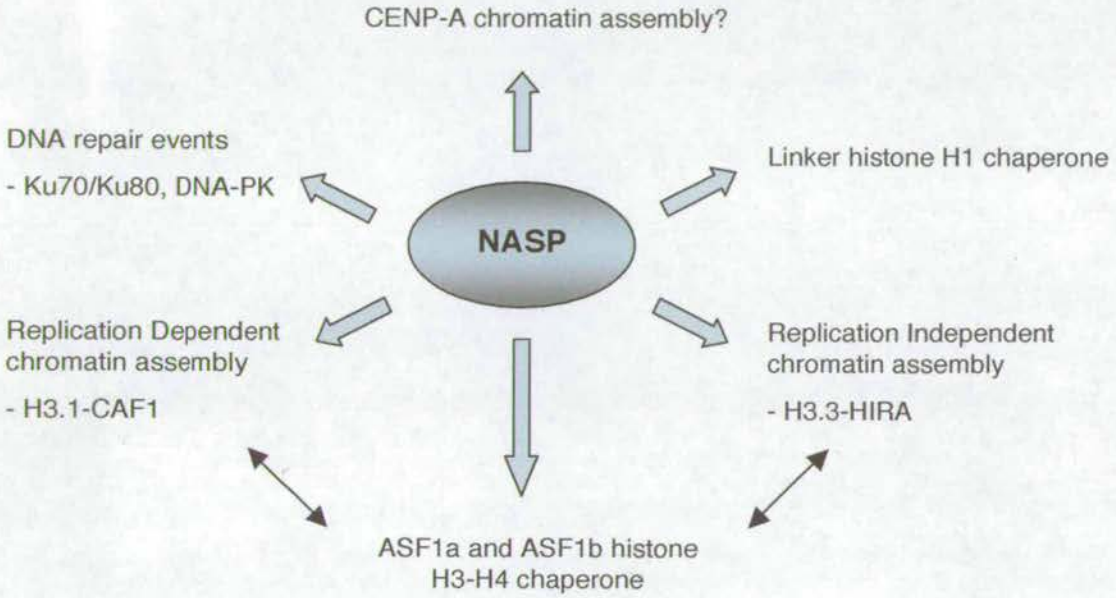


Figure 6-16. Possible roles of NASP in higher eukaryotes.

In HeLa cells, NASP has been described as a chaperone for the linker histone H1 and can transfer H1 onto DNA (Alekseev et al., 2003). The DNA repair proteins Ku70/Ku80 and DNA-PK (protein kinase) were identified as NASP binding partners in HeLa cells by mass spectrometry (Alekseev et al., 2005). Both tNASP and sNASP were found to co-purify with the DNA-replication dependent H3.1-CAF1 deposition complex and with the DNA-replication independent H3.3-HIRA deposition complex isolated in HeLa cells (Tagami et al., 2004). NASP has also been shown to be part of a multi-chaperone complex with ASF1a and AFS1b, which functions to regulate the flow of S phase histones, in particular the replicative histone H3.1, during replicational stress (Groth et al., 2005). ASF1a and ASF1b were found in a complex with both H3.1-CAF1 and H3.3-HIRA (Tagami et al., 2004) and may provide a source of soluble histones, ready for deposition via CAF1 or HIRA mediated pathways. The role of NASP in CENP-A chromatin assembly in higher eukaryotes (other than *D. melanogaster,* as described in this chapter) has not been described.

chaperone complex with the histone chaperone Asf1a and Asf1b, along with the CAF1 components p60 and p48, which together act as a buffer for excess S phase histones produced as a consequence of replicational stress (Groth et al., 2005). The fact that NASP mRNA expression parallels the expression of histone mRNA in the cell cycle, increasing in S phase and declining in G2, further supports a role for NASP in providing a source of histones for deposition into chromatin coupled to replication in mammalian cells (Richardson et al., 2000). In accordance with this, over-expression of tNASP has been shown to delay progression through the cell cycle (Alekseev et al., 2003).

Xenopus N1/N2 is found complexed to non-chromatin bound histone H3 and H4 and plays a role in the storage of excess histone before chromatin assembly takes place in the oocyte (Kleinschmidt et al., 1985). It has also been demonstrated that depletion of N1/N2 from *Xenopus* egg extracts affects nucleosome assembly both in the presence and absence of DNA synthesis (Dilworth et al., 1987, Kleinschmidt et al., 1990). Taken together, it is tempting to speculate that N1/N2 and NASP facilitate proper chromatin assembly in both a replication dependent and independent manner, possibly via co-operating with other histone-chaperones such as CAF1, HIRA and ASF1. The possible roles of NASP and N1/N2 in CENP-A chromatin assembly in higher eukaryotes remain to be fully dissected.

DISCUSSION

Fission yeast is a genetically tractable organism that provides an excellent model of more complex metazoan centromeres, with respect to their composition and general organisation. It is clear that as in higher eukaryotes, fission yeast centromeres are established and maintained in an epigenetic manner and the mere presence of centromeric DNA does not automatically correlate with centromere function (reviewed by Karpen and Allshire, 1997). Centromeres may preferentially form on repetitive DNA, however repetitive DNA is neither necessary nor sufficient for the formation of a functional centromere, and so additional factors must exist which contribute to this essential process. Moreover, as centromeric DNAs are not always necessary for kinetochore formation, this suggests that other regions of the genome may be permissive for centromere activation.

CENP-A is the histone H3 variant found at all eukaryotic centromeres and may act as the primary epigenetic determinant of centromere formation (Palmer et al., 1991, Sullivan et al., 1994). However, many questions regarding CENP-A remain to be answered. For instance, it is not fully understood how CENP-A nucleosomes are assembled and what directs them specifically to the centromere, and only to the centromere. It is also not known whether factors exist that prevent CENP-A from being assembled elsewhere in the genome. In addition, once CENP-A is assembled to form centromeric chromatin, it is not understood how CENP-A is maintained and propagated through subsequent generations. Also, although it has been shown that over-expressed CENP-A can localise to ectopic chromosomal loci that can recruit kinetochore proteins and make microtubule interactions, mis-localised CENP-A does not always trigger functional kinetochore formation (Heun et al., 2006). Thus, it is likely that it is the nature of the underlying chromatin upon which the kinetochore assembles that may provide the contextual specificity of a fully functional centromere. Here, this thesis provides an insight into the role of heterochromatin in centromere formation and function. In addition, this thesis describes the role of a protein required for CENP-A^{Cnp1} localisation to centromeres and possible mechanisms of CENP-A^{Cnp1} chromatin assembly in fission yeast.

TRANSCRIPTIONAL SILENCING AS AN ASSAY OF CENTROMERE FUNCTION

Fission yeast centromeres are composed of two distinct domains that are packaged as silent chromatin: the central domain (*cnt* and *imr*) where CENP-A^{Cnp1} chromatin and the kinetochore proteins assemble and the flanking outer repeat domain (*otr*) that is embedded in heterochromatin. In fission yeast, loss of transcriptional silencing at centromeres can be utilised as an assay of chromatin integrity and centromere function (Allshire et al., 1995,

Ekwall et al., 1995, 1996, 1999, Pidoux et al., 2003). Mutations that alleviate central domain silencing, such as mutations in CENP-A^{Cnp1} itself, are defective in kinetochore assembly (Pidoux et al., 2003, Partridge et al., 2000). Mutations that alleviate outer repeat silencing are defective in heterochromatin formation (Allshire et al., 1995, Ekwall et al., 1995, 1996, 1999). Thus, there is a relationship between the maintenance of silencing at the central domain and the assembly of a functional kinetochore. This correlation can be used as an indirect assay for reporting the efficiency of CENP-A^{Cnp1} incorporation at the central domain. In accordance with this, an additional study has shown that increasing CENP-A^{Cnp1} levels in the cell increases the amount of CENP-A^{Cnp1} that assembles at centromeres (Castillo et al., submitted). This study also demonstrates that the presence of increased CENP-A^{Cnp1} correlates with increased centromere silencing and higher levels of kinetochore proteins are recruited to centromeres (Castillo et al., submitted). Taken together, it is clear that there is correlation between the presence of CENP-A^{Cnp1} at centromeres and transcriptional silencing at centromeres and centromere function.

In chapter 3, *cos* mutants that alleviate both central domain and outer repeat domain silencing were isolated and analysed. The *cos* mutants may uncover a link between the assembly of CENP-A^{Cnp1} / the kinetochore and the integrity of the flanking heterochromatin. Chapters 4 and 5 describe an in-depth analysis of *sim3* mutants that specifically alleviate central core silencing. Sim3 interacts directly with CENP-A^{Cnp1} and it is proposed here that Sim3 acts as a CENP-A^{Cnp1} chaperone. Sim3 is required for the replication independent assembly of CENP-A^{Cnp1} at centromeres during interphase but may contribute to the assembly of CENP-A^{Cnp1} at centromeres at other stages of the cell cycle. Potential mechanisms of replication independent CENP-A^{Cnp1} chromatin assembly are discussed below. In addition, possible roles of Sim3 homologues in higher eukaryotes are described.

POSSIBLE ROLES OF HETEROCHROMATIN AND COS PROTEINS IN KINETOCHORE ASSEMBLY

The presence of heterochromatin at centromeres is a conserved feature of all higher eukaryotic centromeres (for review see Sullivan et al., 2001). One role of heterochromatin is to attract a high density of cohesin to ensure sister chromatids remain tightly cohesed before chromosome segregation at the onset of anaphase (Bernard et al., 2001, Nonaka et al., 2002). Studies carried out in fission yeast using mini-chromosomes have demonstrated that both a portion of the central domain (*cnt*) and at least one outer repeat are required for the formation of a functional centromere that is mitotically stable (Hahnenberger et al., 1991, Takahashi et al., 1992, Baum et al., 1994). This suggests that there may be crosstalk between the central domain region and the outer repeat domain. The *cos* mutants, which are described in chapter 3, alleviate silencing at both the central domain and at outer repeat heterochromatin and may encode proteins which play a role in the interaction between the two domains.

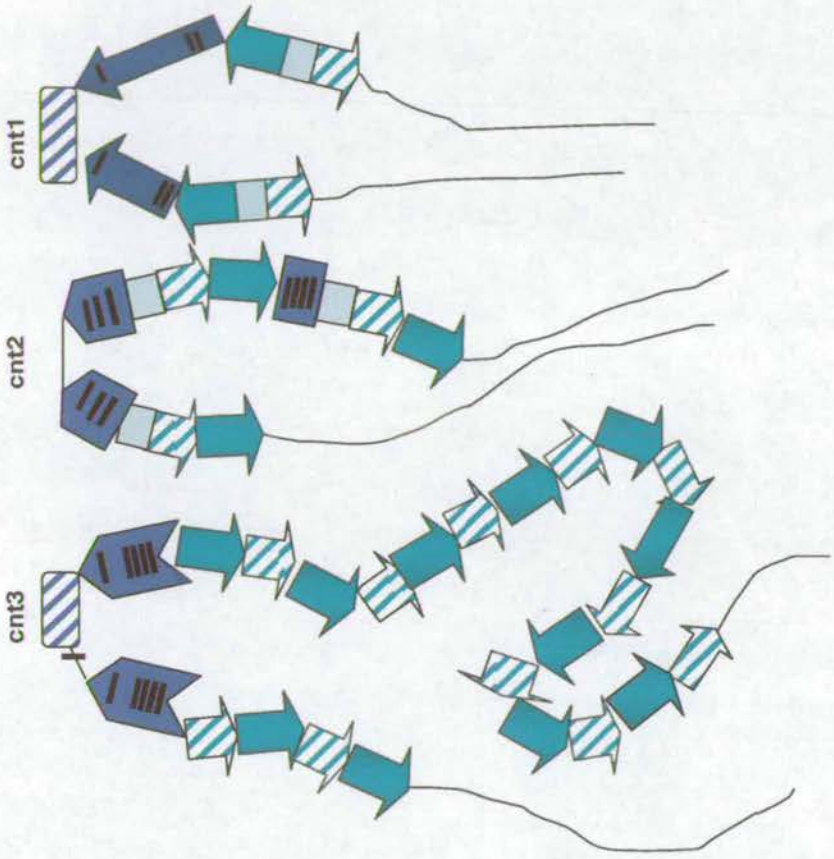


Figure 1: Hypothetical 'loop' organisation of the three fission yeast centromeres (adapted from Takahashi et al., 1992).

The three fission yeast centromeres share the same overall structure, in which a central core (*cnt*) is surrounded by innermost repeats (*imr*) and outer repeats (*otr*). The arrows in the outer repeats (*otr*) represent the repetitive elements *dg* (filled arrows) and *dh* (hatched arrows). The large blue arrows represent the innermost repeats (*imr*). The vertical lines indicate the tRNA genes. *cnt* and *imr* comprise the central domain where CENP-A^{Cnp1} and the kinetochore assembles; *otr* repeats make up the heterochromatin domain. The outer repetitive regions left and right of the central core may interact with each other or with the corresponding regions of other centromeres to form a higher-order complex structure. Interactions between the central domain and the outer repeat domain may be mediated by proteins such as those encoded by the cos^+ genes. In this way the central core region may be presented in the correct configuration to facilitate CENP- A^{Cnp1} assembly and to achieve proper microtubule attachments required for accurate segregation of chromosomes in mitosis.

As discussed in chapter 3, the *cos*$^+$ genes may encode factors that are required to set up the proper architecture of the centromere that is required for function. Due to the inverted repeat structure of the heterochromatin that flanks the central domain of *S. pombe* centromeres, it has been proposed that *S. pombe* centromeres may form a looped higher-order structure (Takahashi et al., 1992, Clarke et al., 1993). It is possible that this higher order structure leads to the compaction of the repetitive sequences that comprise the centromere and this contributes to the formation of silent and repressed chromatin at the two domains (see Discussion Figure 1). In this way, the tandem inverted repeat structure at the centromere may interact directly or via centromere binding proteins and mediate their own repression; mutants defective in this structure may lose silencing of these specialised chromatin domains. Indeed, strict mechanisms appear to be in place to ensure that centromeric sequences are perfectly symmetric as sequencing of *imrL* and *imrR* from different *S. pombe* isolates has shown that polymorphisms occur at corresponding positions on both sides of the centromere (Takahashi et al., 1992). In addition both the position and orientation of the central core and *imr* with respect to the outer repeats was found to differ in different laboratory strains (Steiner and Clarke, 1994), suggesting that there may be specific and controlled recombination events that occur between the central domain and outer repeat domain at centromeres.

Interactions may also occur between the central domain and the outer repeat domain of an individual centromere or between the inverted outer repetitive regions on the left and right sides of an individual centromere. These interactions may be mediated by proteins, such as those encoded by *cos*$^+$ genes that alleviate silencing at both central and heterochromatin domains, presumably due to disrupted chromatin at both domains. It is also possible that *cos*$^+$ genes encode chromatin-remodelling factors that modify the integrity of chromatin at centromeres. In addition, interactions may occur between the central domain and outer repeat domain of centromeres on different chromosomes, clustering the three centromeres together in a conformation that facilitates robust capture of spindle microtubules in mitosis. The interaction between the two domains may also lead to the formation of a structurally distinct conformation at the centromere that distinguishes this specialised region of the chromosome from the rest of the genome. Another possibility is that heterochromatin at the centromere may act to present the central domain in the correct configuration to promote CENP-A^{Cnp1} and kinetochore assembly. The action of the histone methyl transferase Clr4 leads to heterochromatin assembly, as it is required to methylate histone H3 on lysine 9 and this provides a binding site for Swi6, the heterochromatin protein 1 (HP1) homologue (Rea et al., 2000, Nakayama et al., 2001). In this way, heterochromatin proteins such as Clr4 and Swi6 may be required to provide a more suitable environment that specifies and maintains the site of CENP-A^{Cnp1} chromatin assembly through the cell cycle.

Whether this looped model can be extrapolated to the larger and more complex centromeres of higher eukaryotes remains to be determined. In mammalian cells, a repeat subunit model was first proposed by Zinkowski et al. (1991), suggesting that the centromere/kinetochore plate-like structure visible by EM on eukaryotic chromosomes is formed by the folding of a linear DNA fibre consisting of tandemly repeated subunits. Recent studies in human and flies also suggest that the centromere is organized into a three dimensional structure which acts as another mark to give a centromere its identity (Sullivan and Karpen, 2004, Schueler and Sullivan, 2006). These models suggests that a higher-ordered structure is formed at centromeres by the spiralling of centromeric DNA like a solenoid or by looping of centromeric DNA which folds back on itself as is suggested in fission yeast. According to this model, CENP-A nucleosomes coalesce and are positioned toward the outer face of the mitotic chromosome to facilitate the recruitment of kinetochore proteins and to enable centromeres to make proper connections with spindle microtubules emanating from the poles. These CENP-A nucleosomes sit on a bed of heterochromatin, which lies both underneath the CENP-A domain and flanking the CENP-A domain on either side. As the nucleosomes containing histone H3 that are found dispersed within the CENP-A chromatin domain are always in the H3K4me2 state, it is thought that the combination of post-translational modifications of core histones together with CENP-A nucleosomes may also contribute to proper kinetochore formation. This specialised chromatin, called 'centrochromatin', may provide a mark that distinguishes centromeric chromatin from the rest of the genome (Sullivan and Karpen, 2004, reviewed by Dunleavy et al., 2005, see Chapter 1 Figure 1-8). In this way, heterochromatin contributes structurally to the formation of a conformationally more robust centromere that may provide a signal to indicate CENP-A chromatin assembly. Although the role of/requirement for H3K4me2 at centromeres has not been tested, as H3K4me2 is a marker of active transcription, it is possible that the transcriptional machinery plays a role in establishing proper centromeric chromatin. For example, the process of transcription may allow chromatin at centromeres to be in a more 'open' state to facilitate CENP-A incorporation.

POSSIBLE ROLES OF MCL1 AT CENTROMERES

In chapter 3, five alleles of *cos1* were isolated in a screen designed to identify mutants that alleviate silencing at both the central domain and at outer repeat heterochromatin. *cos1*$^{+}$ was found to be allelic to *mcl1*$^{+}$ (mini-chromosome loss 1), which encodes a protein that was previously shown to be required for efficient chromosome segregation, for efficient DNA replication and for the maintenance of sister chromatid cohesion (Williams et al., 2002). The analysis presented here shows that temperature sensitive mutations in *cos1* give rise to alleviation of silencing at both the central domain and at the flanking heterochromatin at centromeres. Alleviation of silencing at the central domain is often correlated with loss of CENP-A^{Cnp1} from centromeres and alleviation of silencing at the outer repeat domain is

often correlated with loss of the heterochromatin marker H3K9me2 (Pidoux et al., 2003, Ekwall et al., 1999). In *cos1* mutants, however, both the localisation of CENP-A^{Cnp1} at centromeres and the levels of the heterochromatin marker H3K9me2 appear to be unperturbed. A recent report however, has demonstrated that CENP-A^{Cnp1}-GFP is delocalised and is dispersed throughout the nucleus in *mcl1Δ* cells (Mamnun et al., 2006), suggesting that Mcl1 is required for the localisation of tagged CENP-A^{Cnp1} to centromeres. This result also suggests that temperature sensitive mutations in *mcl1* may not be fully penetrant even at the restrictive temperature or that mutant Mcl1 is sufficient to retain CENP-A^{Cnp1} at centromeres. *mcl1Δ* cells were previously reported to be extremely slow growing even at permissive temperature (Williams et al., 2002, Tsutsui et al., 2005) and due to this reduced cell viability it is possible that *mcl1Δ* cells may report complex or indirect effects. Mamnun et al. (2006) also demonstrated that *mcl1Δ* cells displayed defective silencing at outer repeat heterochromatin and not at the telomeric region, which is consistent with data presented for the temperature sensitive alleles of *mcl1* isolated in chapter 3. Taken together, the fact that loss of functional Mcl1 gives rise to alleviation of both central domain and outer repeat silencing, coupled with the observation that CENP-A^{Cnp1}-GFP appears to be delocalised in an *mcl1*$^{+}$ deleted strain, suggests the integrity of the neighbouring heterochromatin at centromeres may influence the assembly of CENP-A^{Cnp1} at the central domain. It is also possible that Mcl1 facilitates the cross talk between the central domain and flanking heterochromatin by contributing to the formation of a DNA looping structure at the centromere as proposed above.

Mcl1, like its budding yeast homologue Ctf4p, has been shown to bind to the replicative polymerase Polα and to display synthetic lethal interactions with several replication components (Williams et al., 2005, Miles and Formosa, 1992). These findings suggest a role for Mcl1 in DNA replication. In addition, Mcl1 is localised to chromatin during G1 and S phase and cells lacking functional *mcl1*$^{+}$ are dependent on the DNA damage checkpoint protein Rad3 for survival, further implicating a role for Mcl1 at replication, particularly during replication stress (Williams et al., 2005). Deletion or disruption of Mcl1 also gives rise to segregation defects such as lagging chromosomes, as described in this thesis, and to defects in sister chromatin cohesion as previously reported (Williams et al., 2002, Tsutsui et al., 2005). As defective *mcl1*$^{+}$ also causes alleviation of silencing at centromeres, it is possible that one function of Mcl1 may be to ensure silent chromatin marks such as H3K9me2 are maintained after passage of the replication fork as centromeric DNA is replicated during S phase (refer to Chapter 3, Figure 3-13A). This may involve maintaining the heterochromatin marker H3K9me2 at a sufficient level to recruit sufficient Swi6 to ensure robust sister centromere cohesion. It is also possible that Mcl1 may help to establish or maintain the integrity of CENP-A^{Cnp1} nucleosomes in the wake of DNA replication at the central domain. For this reason, it would be interesting to test whether newly synthesized CENP-A^{Cnp1}-GFP can localise to centromeres in cells lacking functional Mcl1 at different

stages of the cell cycle such as in S phase.

Budding yeast Ctf4p has also been shown to inhibit the binding of the chromatin remodelling complex FACT (Facilitates Chromatin Transcription) to Polα (Wittmeyer and Formosa, 1999). During transcription, FACT has been shown to act to destabilise the nucleosome, such that one H2A-H2B dimer is removed to facilitate the passage of the RNA polymerase II (Orphanides et al., 1999, Berlotserkovskaya et al., 2003). FACT is also involved in the reassembly of an H2A-H2B dimer back into the nucleosome after transcription has occurred (Orphanides et al., 1999, Berlotserkovskaya et al., 2003). Although a role for FACT in replication has not been established, it is possible that through its interaction with Polα, FACT may facilitate the passage of the DNA polymerase through chromatin, similar to its role in transcription. As Ctf4p appears to regulate the accessibility of the FACT to Polα, it is possible that Mcl1 may act to regulate FACT remodelling activity at the centromere at either replication or during transcription. Loss of functional *mcl1*$^{+}$ could result in aberrant chromatin remodelling during replication or transcription at the centromere by FACT. This may result in the loss of heterochromatin/kinetochore integrity or some other changes in chromatin structure that is displayed as a loss of silencing at both the central domain and heterochromatin domain. Interestingly, both FACT components FACTp140 and FACTp80 have been identified in a complex with CENP-A from HeLa cells (Obuse et al., 2004, Foltz et al., 2006). Early EM observations by Rieder (1979) suggested the presence of an RNAse sensitive component at the kinetochore. More recent evidence from maize, rice and mice has now implicated transcription in the assembly of the kinetochore (Topp et al., 2005, Bouzinba-Segard et al., 2006, Nagaki et al., 2004). It is possible that alterations of FACT activity may disrupt the integrity of the chromatin at centromeres and gives rise to the inefficient assembly of CENP-A^{Cnp1} chromatin (refer to Chapter 3, Figure 3-13B).

HISTONE VARIANTS SPECIFY ALTERNATE CHROMATIN STATES

It is clear that chromatin can be altered on a number of levels which affect the read out of the information stored in the underlying DNA; be it through the assembly of specific histone variants through specialised deposition machinery, through the establishment of histone modifications or by the binding of additional chromatin modifying proteins (reviewed by Polo and Almouzni, 2006). It is apparent that highly conserved histones have diverged through evolution to give rise to histone variants that provide another mechanism to alter chromatin states, be it transcriptional repression, activation or to designate a particular role such as centromere formation (reviewed by Malik and Henikoff, 2003). It is tempting to speculate that for each histone variant a specific chaperone and deposition factor exists. Exactly what determines the specificity of these factors also remains to be determined. Here, the role of fission yeast Sim3 that may act as a CENP-A^{Cnp1} chaperone

and facilitate the delivery of this histone H3 variant to centromeres has been described. Exactly how Sim3 orchestrates the deposition of CENP-A^{Cnp1} specifically at this region of the genome remains to be investigated.

It is known that bulk histones are upregulated at S phase and incorporated into chromatin in a replication dependent manner (reviewed by Krude and Keller, 2001). Histone variants or 'replacement' histones, such as H3.3, are constitutively expressed throughout the cell cycle and are deposited in a replication independent manner (reviewed by Henikoff et al., 2002). Thus, replication dependent and independent mechanisms of histone assembly provide another means of specifying an alternative chromatin state. It is interesting to note that only the H3.3 counterpart is present in both budding and fission yeast, which is capable of both replication dependent and independent assembly in these organisms (reviewed by Malik and Henikoff, 2003). It is now known in mammalian cells that the histone H3 variant CENP-A can be deposited at the centromere in the absence of DNA replication indicating that centromere specific incorporation is not coupled to S phase (Shelby et al., 2000, Sullivan and Karpen, 2001). Similarly, the incorporation of CENP-A in G2 has been demonstrated in fission yeast and *Arabidopsis thaliana* (Takahashi et al., 2005, Lermontova et al., 2006). It is possible that CENP-A chromatin assembly is a highly dynamic process and a constant replacement of CENP-A occurs throughout all stages of the cell cycle, which may coincide with DNA replication but is not strictly coupled to it.

SIM3 AND CENP-A^{Cnp1} CHROMATIN ASSEMBLY

As discussed above, it is not fully understood how CENP-A is assembled into centromeric chromatin or how it is maintained at centromeres once CENP-A chromatin is established. Although studies in fission yeast have identified a number of factors required for the localisation of CENP-A at centromeres, the exact mechanism and additional factors required to facilitate this process remain to be elucidated. A number of CENP-A interacting proteins have been identified in human cells but the role of these proteins in CENP-A chromatin assembly awaits further investigation (Obuse et al., 2004, Foltz et al., 2006). In chapter 4 and 5, the role of the Sim3 (which shows homology to conserved histone-binding proteins N1/N2 and NASP) in the assembly of CENP-A^{Cnp1} is characterised. *sim3* mutants specifically alleviate silencing at the central core region, where CENP-A^{Cnp1} and the kinetochore proteins are known to assemble, and also display defects in chromosome segregation. Sim3 is a nuclear protein that physically interacts with CENP-A^{Cnp1} and its sequence homology to conserved histone chaperones is consistent with the proposed role of Sim3 as a CENP-A^{Cnp1} chaperone. In chapter 5, it was demonstrated that Sim3 is required for the localisation of CENP-A^{Cnp1} at centromeres. Moreover, Sim3 was shown to be required for the localisation of newly synthesized CENP-A^{Cnp1} to centromeres at the G2 phase of the cell cycle.

Models of Sim3 assisted CENP-A chromatin assembly

Possible mechanisms of Sim3 assisted CENP-A^{Cnp1} chromatin assembly are discussed below. It is also possible that Sim3 contributes to the assembly of CENP-A^{Cnp1} at other stages of the cell cycle and is not restricted to assembly in G2. For example, Sim3 or associated factors may act also in S phase to deliver CENP-A^{Cnp1} to the replication fork for incorporation into chromatin at the same time as the canonical histones. According to this mechanism, Sim3 or associated factors would need to specifically recognise when centromeric DNA is being replicated in S phase and may be actively recruited to the replication fork to enable CENP-A^{Cnp1} deposition. The mode of Sim3 assisted CENP-A^{Cnp1} chromatin may also be a combination of the following mechanisms and each model is not exclusive. Sim3 may also act in conjunction with other factors shown previously to be required for proper CENP-A^{Cnp1} localisation.

Model 1: Sim3 may act as a CENP-A^{Cnp1} chaperone

Sim3 may be acting as a chaperone or 'escort' that protects the CENP-A^{Cnp1} pool in the cell, keeping it in a ready state for its delivery to the centromere at in a replication-dependent or replication-independent manner.

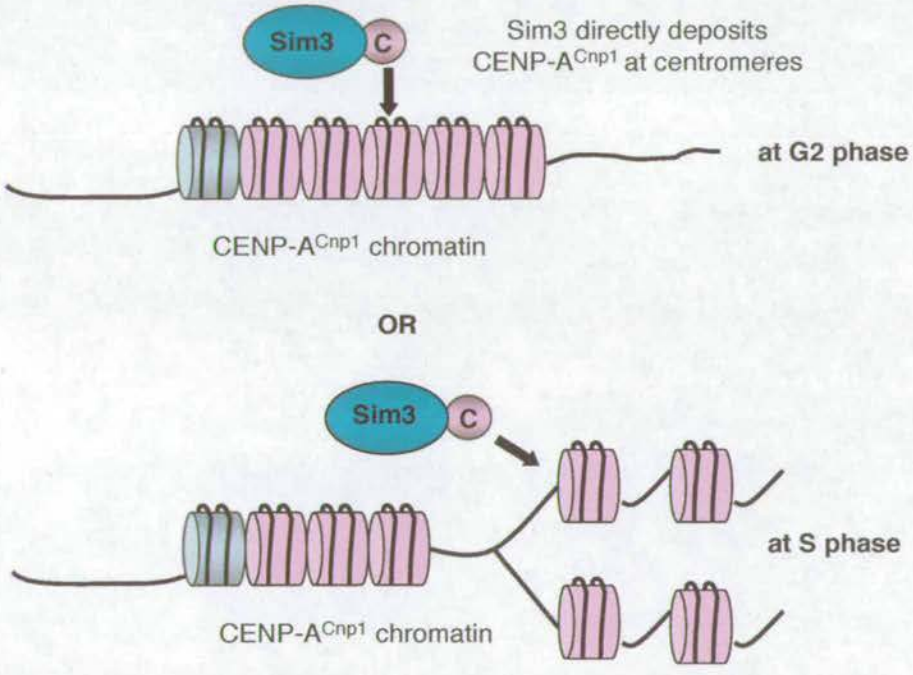
Model 1A: CENP-A recognition model

Sim3 may deliver CENP-A^{Cnp1} to the centromere and may play a direct role in its assembly into chromatin. Sim3 is an abundant nuclear protein. However, despite a variety of efforts, evidence that Sim3 is specifically associated with the centromere was not obtained. Sim3 may however only need to associate transiently with the centromere at certain stages of the cell cycle and within a very narrow window of time in order to deposit CENP-A^{Cnp1} there. It is possible that CENP-A^{Cnp1} that is already at centromeres may provide the mark for where to put more CENP-A^{Cnp1}. In this way, perhaps existing CENP-A^{Cnp1} can target newly synthesized CENP-A^{Cnp1} that is bound to Sim3 to centromeres. Sim3 may only be involved in the process of CENP-A^{Cnp1} delivery, for example, by protecting CENP-A^{Cnp1} from degradation or preventing it from being mis-incorporated elsewhere. This mechanism however only accounts for how CENP-A^{Cnp1} is propagated through cell cycle generation but does not explain how CENP-A^{Cnp1} is first established specifically at centromeres.

Model 1B: Adapter recognition model

As an alternative to a direct role for Sim3 in assembly of CENP-A^{Cnp1}, it is possible that Sim3 passes CENP-A^{Cnp1} to an adapter or factor X that specifically recognises the centromere as the site of CENP-A^{Cnp1} chromatin assembly at either G2 or S phase or other stages of the cell cycle. It is possible that factor X is a chromatin assembly factor that is directly associated

MODEL 1A

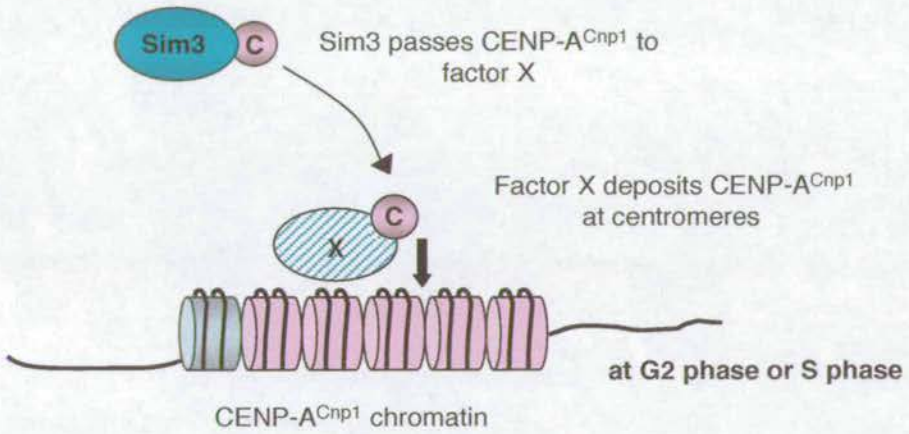


Models of Sim3 assisted CENP-A^{Cnp1} chromatin assembly.

MODEL 1A: CENP-A recognition model

Sim3 could be acting as chaperone or 'escort' that protects the CENP-A^{Cnp1} pool in the cell, keeping it ready state for delivery to the centromere. Sim3 may directly deposit CENP-A^{Cnp1} at centromeres during S phase or G2 or other stages of the cell cycle. It is possible that existing CENP-A^{Cnp1} at centromeres directs the recruitment of new CENP-A^{Cnp1} to centromeres. Grey cylinders represent nucleosomes containing H3, purple cylinders represent nucleosomes containing CENP-A^{Cnp1}. C=CENP-A^{Cnp1}.

MODEL 1B



Models of Sim3 assisted CENP-A^{Cnp1} chromatin assembly.

MODEL 1B: Adaptor recognition model

Sim3 could be acting as a CENP-A^{Cnp1} chaperone and may pass CENP-A^{Cnp1} to an adapter or factor X that specifically recognizes the centromere as the site of CENP-A^{Cnp1} chromatin assembly. Factor X could be a remodelling factor that is sitting at the centromere and may act to assemble CENP-A^{Cnp1} chromatin at G2, S or other stages of the cell cycle (see text for details). It is also possible that existing CENP-A^{Cnp1} at centromeres directs the recruitment of new CENP-A^{Cnp1} to centromeres via factor X. Grey cylinders represent nucleosomes containing H3, purple cylinders represent nucleosomes containing CENP-A^{Cnp1}. C=CENP-A^{Cnp1}.

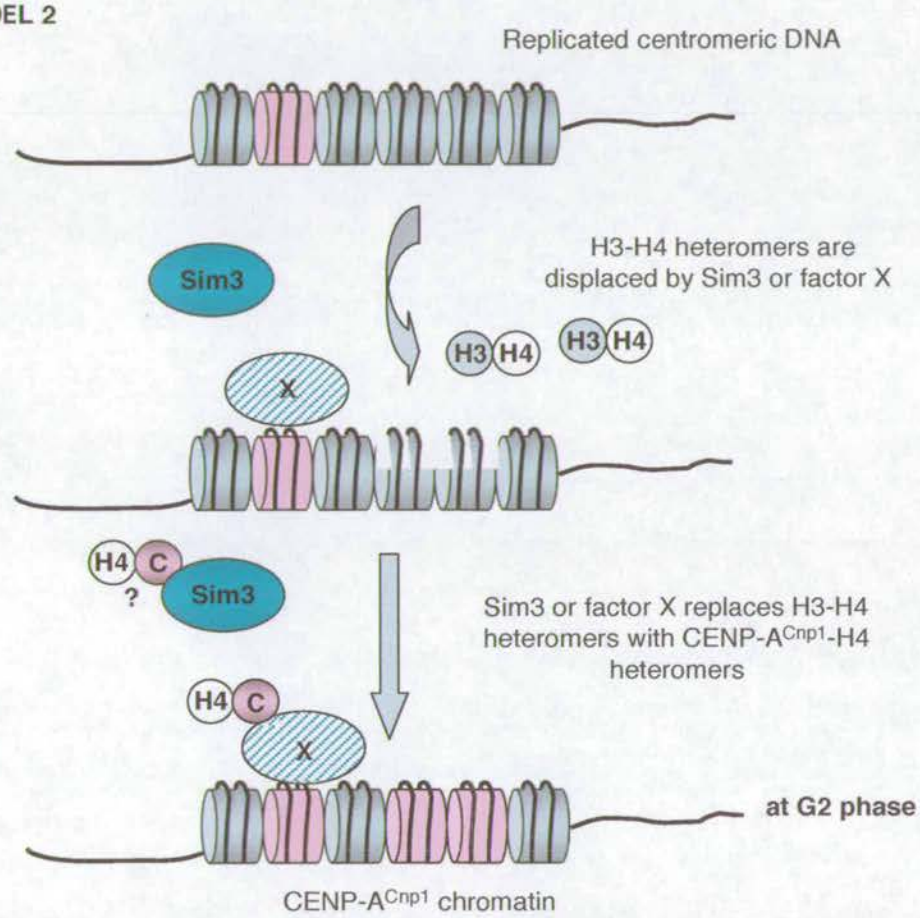
with centromeric chromatin and may possess chromatin-remodelling activity. As discussed in model 1A, unincorporated CENP-A^{Cnp1} that is bound to Sim3 may be recognised by a factor X positioned at the centromere that receives the newly synthesized CENP-A^{Cnp1} and incorporates it. For this reason, it would be of interest to determine if Sim3 is part of a larger complex and to decipher the function of such putative Sim3 interacting partners. Interestingly, *sim1* mutants were isolated from the *sim* screen to identify mutants that specifically alleviate central core silencing (A. Pidoux, unpublished result). Sim1 shows weak homology to Scm3p/NAP1, localises specifically to the kinetochore and *sim1* mutants have reduced CENP-A^{Cnp1} associated with centromeres (J. Abbott, PhD Thesis, University of Edinburgh, 2004). Therefore, it is plausible that Sim1 or the genes encoded by *sim5* or *sim7* that were isolated from the *sim* screen and also specifically alleviate central core silencing could represent assembly factor X.

Model 2: Sim3 may act in G2 to facilitate the replacement of H3 with CENP-A^{Cnp1}.

In chapter 5, *sim3* mutants were observed to have increased histone H3 and reduced CENP-A^{Cnp1} associated with centromeres. It has recently been observed in fission yeast that in the presence of excess CENP-A^{Cnp1}, more CENP-A^{Cnp1} can be deposited at the centromere at the expense of histone H3 (Castillo et al., submitted). Castillo et al. have also demonstrated that an excess of histone H3 allows the deposition of H3 in the place of CENP-A^{Cnp1} at centromeres and this leads to subsequent defects in chromosome segregation. These results suggest that the histone composition at the centromere is malleable and may be subject to replacement or remodelling depending on the histones that are available for deposition. Therefore, it is possible that Sim3, or a centromere specific assembly factor X, may act in G2 to strip out H3 or participate in the exchange of H3 that was deposited in S phase and replace it with CENP-A^{Cnp1}. According to this model, CENP-A chromatin is randomly distributed to both daughter strands during replication and H3 nucleosomes are incorporated as temporary 'placeholders' to mark the site of CENP-A^{Cnp1} assembly (Shelby et al., 2000, Sullivan, 2003). In the subsequent G2 phase, temporary H3 nucleosomes are removed and are replaced with *de novo* synthesized CENP-A^{Cnp1} nucleosomes, possibly by using the parental CENP-A^{Cnp1} as a template to mark the new sites of incorporation. It is not known whether Sim3 can also associate with histone H4 *in vivo*, however it is possible that H3 could be replaced with CENP-A^{Cnp1} in a process that involves the incorporation of CENP-A^{Cnp1}-H4 heteromers in the place of H3-H4 heteromers. These heteromers might be heterotetramers or perhaps heterodimers, as it was demonstrated in HeLa cells that H3.1-H4 and H3.3-H4 are deposited as dimers and not as tetramers as previously understood (Tagami et al., 2004).

An interesting experiment to test this model could utilise a strain that contains a large insertion of non-centromeric DNA (4.7 kb) at the central core that can assemble some

MODEL 2



Models of Sim3 assisted CENP-A^{Cnp1} chromatin assembly.

MODEL 2:

Sim3 may act in G2 to strip out H3 as H3-H4 heteromers that were deposited in S phase and replace them with CENP-A^{Cnp1}-H4 heteromers. Sim3 may play a direct role the deposition of CENP-A^{Cnp1} or may pass CENP-A^{Cnp1} to a factor X that specifically recognises the centromere and incorporates it there. Sim3 or factor X may also participate in the removal of H3-H4 heteromers. Grey cylinders represent nucleosomes containing H3, purple cylinders represent nucleosomes containing CENP-A^{Cnp1}. C=CENP-A^{Cnp1}.

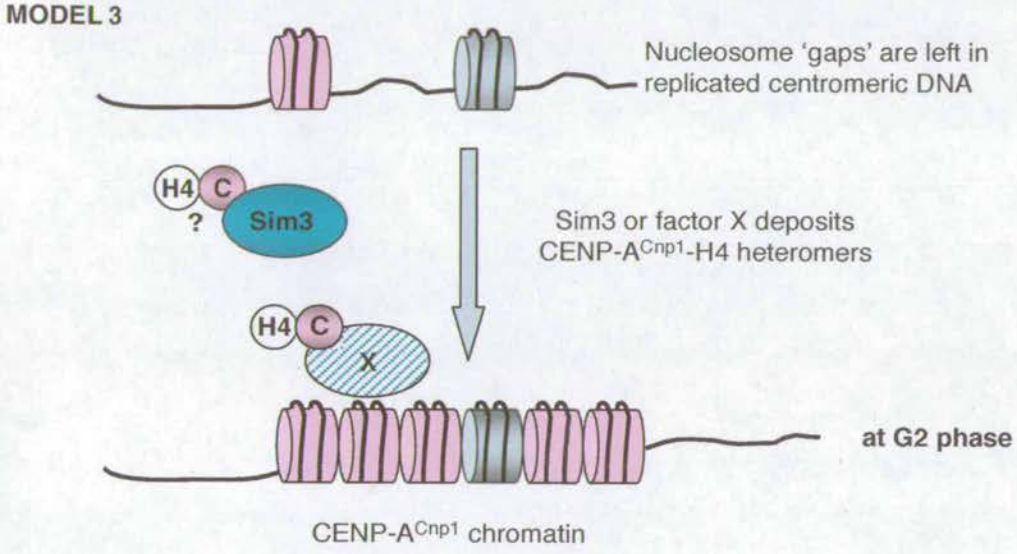
CENP-A^{Cnp1} chromatin but assembles more H3 chromatin (A. Castillo, submitted). This provides a way of loading the centromere with more H3 than normal and the cells could then be arrested in G2 using the *cdc25-22* mutation. If new synthesis of GFP-tagged CENP-A was then induced in G2 using the invertase promoter, perhaps the replacement of H3 with tagged CENP-A could be monitored by chromatin immunoprecipitation. This experiment would test the idea that H3 is replaced by the histone H3 variant CENP-A^{Cnp1} at centromeres in a manner that is independent of replication. A similar mechanism of exchange of H2A for the H2A.Z variant has been demonstrated *in vitro*, where H2A-H2B dimers are replaced by the H2A.Z-H2B dimers by the budding yeast SWR1 chromatin remodelling complex (Mizuguchi et al., 2003). In *Drosophila* and human cells, H3.3 is found to replace H3 upon transcriptional activation (Ahmad and Henikoff, 2002, Janicki et al., 2004, Schwartz and Ahmad, 2005), which may be mediated by the DNA replication independent nucleosome assembly factor HIRA (Ray-Gallet et al., 2002, Tagami et al., 2004). Thus, the exchange of canonical histones for histone variants independently of replication provides another mechanism of specifying an alternate chromatin state such as the site of kinetochore formation and centromere activation.

Model 3: Sim3 may act in G2 to fill gaps left in centromeric chromatin for CENP-A^{Cnp1}.

It has also been proposed that as centromeric DNA is replicated in S phase, 'gaps' are left for CENP-A^{Cnp1} nucleosomes, which are filled by CENP-A^{Cnp1} nucleosomes in G2 (Sullivan, 2001). It is possible that Sim3 or assembly factor X may facilitate the delivery of CENP-A^{Cnp1} molecules for centromeric chromatin assembly after DNA replication has occurred. According to this model, gaps occur behind the replication fork as parental CENP-A nucleosomes are partitioned randomly between the two daughter strands. This suggests that there may also be mechanisms in place to prevent the assembly of canonical histones at centromeric DNA during replication. Another possibility is that the DNA replication machinery has a mechanism of detecting 'special' centromeric chromatin in S phase and signals chromatin assembly factors to leave nucleosome gaps in the chromatin. Pre-existing CENP-A^{Cnp1} nucleosomes may serve as the recognition factor that promotes the specific assembly of additional nucleosomes containing newly synthesized CENP-A^{Cnp1} in G2 phase. Furthermore, newly synthesized CENP-A^{Cnp1} that is complexed to Sim3 or assembly factor X may recognise parental CENP-A^{Cnp1} nucleosomes. Here, Sim3 may act by keeping CENP-A^{Cnp1} in protected state before it is deposited post replication to fill spaces left for new CENP-A^{Cnp1} nucleosomes.

Other possible mechanisms of replication independent CENP-A chromatin assembly

Although it is clear that Sim3 plays an important role in the assembly of CENP-A^{Cnp1} chromatin during G2, it is possible that Sim3 contributes to CENP-A^{Cnp1} chromatin



Models of Sim3 assisted CENP-A^{Cnp1} chromatin assembly.

MODEL 3:

Sim3 may act in G2 after DNA replication has occurred, to fill gaps that may be left in centromeric chromatin during S phase with newly synthesized CENP-A^{Cnp1}, possibly as CENP-A^{Cnp1}-H4 heteromers. Alternatively Sim3 passes CENP-A^{Cnp1} to factor X that recognises the centromere and deposits new CENP-A^{Cnp1} nucleosomes in G2. Grey cylinders represent nucleosomes containing H3, purple cylinders represent nucleosomes containing CENP-A^{Cnp1}. C=CENP-A^{Cnp1}.

assembly at other stages of the cell cycle. Henikoff and Dalal (2005) have proposed the possibility that gaps in centromeric chromatin may occur at anaphase due to the tension imposed by the attached spindle microtubules. According to this model, H3 nucleosomes temporarily placed at the centromere during S phase are unravelled during the subsequent mitosis by the pulling forces of the spindle microtubules, whereas CENP-A nucleosomes are retained at the centromere as they can withstand the tension (Henikoff and Dalal, 2005). Indeed, it has been demonstrated biochemically that CENP-A-H4 containing nucleosomes are conformationally more rigid in nature than H3-H4 containing nucleosomes (Black et al., 2004). In this model, centromeric DNA wrapped around temporary H3 nucleosomes unravels more easily and CENP-A is deposited in its place. A related mechanism has been proposed by Mellone and Allshire (2003). This model suggests that it is the presence of a functional kinetochore under the tension exerted by proper microtubule attachments, which generates the epigenetic 'put CENP-A here' signal in the following cell cycle. Preliminary results using the inducible invertase promoter in a *cdc10* mutant background suggests that newly produced GFP-CENP-A^{Cnp1} is not efficiently targeted to centromeres at G1 stage of the cell cycle (E. Dunleavy, unpublished observation). However, a more thorough examination of when exactly CENP-A^{Cnp1} chromatin assembly can occur, or indeed, is prevented throughout the cell cycle needs to be performed.

ROLE OF Sim3 IN HIGHER EUKARYOTES

Sim3 is a highly conserved protein that shows homology to the histone binding proteins *Xenopus laevis* N1/N2 and NASP. N1/N2 is a histone H3 and H4 binding protein that can transfer the histones to DNA to facilitate chromatin assembly *in vitro* (Kleinschmidt et al., 1985; Kleinschmidt et al., 1990). Mammalian NASP (nuclear autoantigenic sperm protein) is a histone H1 binding protein that facilitates the transport of the linker histone H1 into the nuclei of dividing cells and can transfer H1 to DNA *in vitro* (Richardson et al., 2000, (Alekseev et al., 2003). More recently NASP has been shown to interact with histone H3 and is present in both the H3.1-CAF1 and H3.3-HIRA multi-chaperone nucleosome remodelling complexes (Tagami et al., 2004). CAF1 facilitates replication dependent nucleosome assembly, whereas HIRA facilitates replication independent nucleosome assembly (Tagami et al., 2004, Smith and Stillman, 1989, Ray-Gallet et al., 2002). The role of *Xenopus* N1/N2 and human NASP in CENP-A chromatin assembly has previously not been addressed. Analysis carried out in chapter 6 demonstrates that *Drosophila* DmNASP is not required for the localisation of either endogenous or GFP tagged CENP-A^{CID} to centromeres in fly S2 tissue culture cells. Additional experiments (carried out in collaboration with B. Mellone and G. Karpen, UC, Berkeley) demonstrated that newly synthesized GFP-CENP-A^{CID} did not show any defects in import of GFP-CENP-A^{CID} into the nucleus or mislocalisation to other chromatin sites. Whether DmNASP is required for the localisation of newly synthesized CENP-A^{CID} to centromeres remains to be investigated. As the

incorporation of CENP-A into chromatin is an essential process, it is likely that there are a number of proteins that contribute to this process in higher eukaryotes and that loss of DmNASP may be compensated by the action of other factors that contribute to CENP-A^{CID} chromatin assembly in flies.

A weak interaction was also detected *in vitro* between DmNASP and histone H3.3, suggesting that the role of DmNASP in *Drosophila* may be in H3 chromatin assembly and not tailored torward the histone H3 variant CENP-A^{CID}, as it is in fission yeast. It is tempting to speculate that DmNASP may be involved in the replication independent assembly of H3.3 chromatin, as human NASP was also isolated as part of the HIRA complex (Tagami et al., 2006). However, this interaction between DmNASP and H3.3 would need to be confirmed *in vivo*. The presence of mammalian NASP in the CAF1 complex implies that NASP may have a role in canonical H3.1 deposition and the completion of S phase in the cell cycle (Tagami et al., 2004). Human NASP has also been isolated in a discrete multi-chaperone complex with the histone chaperones Asf1a and Asf1b, along with the CAF1 components p60 and p48, which together act as a buffer for excess S phase histones produced as a consequence of replicational stress (Groth et al., 2005). NASP has also been found to be a binding partner of Ku70/Ku80 and DNA-PK in HeLa cells, that are necessary for DNA double strand break repair by homologous end joining, indicating that NASP may play an additional role in DNA repair events (Alekseev et al., 2005). From the recent finding that *NASP*$^{-/-}$ null mice are embryonic lethal, it is clear that NASP plays a very important role in higher eukaryotes (Richardson et al., 2006). Taken together, NASP may contribute to a number of pathways including DNA replication and repair, the assembly of H3.1 in S phase via CAF1, the assembly of H3.3 via HIRA, by acting as a histone buffer in conjunction with ASF1 and in the transport and deposition of histone H1 (refer to Chapter 6, Figure 6-16).

CONCLUSIONS AND PERSPECTIVES

A common feature of most eukaryotic centromeres is the presence of heterochromatin, accompanied by the presence of the histone H3 variant CENP-A. Fission yeast centromeres resemble vertebrate centromeres in that they are composed of a central domain, where CENP-A^{Cnp1} and the kinetochore assemble, which is embedded in repetitive heterochromatin. The *cos* mutants described here may give insight into interactions between the central domain and outer repeat heterochromatin. *cos1*$^{+}$ is allelic to *mcl1*$^{+}$ and Mcl1 may function to ensure that the features of silent centromeric chromatin, such as the association of CENP-A^{Cnp1} and correct levels of Swi6/cohesion at outer repeats, are retained after replication. It is also proposed here that Sim3 acts as a CENP-A^{Cnp1} chaperone that ensures the delivery of CENP-A^{Cnp1} to centromeres and only to centromeres. Sim3 is required for the replication independent assembly of CENP-A^{Cnp1} chromatin in interphase

but it is possible that Sim3 contributes to the assembly of CENP-A ^{Cnp1} at other cell cycle stages. Analysis of fission yeast Sim3 described in this thesis, suggests a novel role of histone chaperone NASP in CENP-A chromatin assembly at vertebrate centromeres. It is tantalizing to speculate that the role of NASP as a CENP-A chaperone is conserved in higher eukaryotes. The requirement for NASP in orchestrating the deposition of newly synthesized CENP-A at centromeres remains to be investigated in flies and other metazoa.

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Centromeric chromatin makes its mark

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Post-translational modifications of core histones, particularly in the N terminus, seem to define different chromatin states in the genome. For instance, actively transcribed regions of euchromatin have a completely different set of 'marks' compared with silent heterochromatin. Recent analyses surprisingly demonstrate that, in addition to containing the histone H3 variant CENP-A, the centromeric chromatin that underlies the kinetochore bears a distinct combination of histone H3 modifications.

Introduction

Chromosomal DNA is wrapped around an octamer of proteins comprising two subunits of each histone H3, H4, H2A and H2B to form a nucleosome [1]. The charged N-terminal histone 'tails' protrude from the nucleosome and these, in addition to other residues, are subject to a diverse array of post-translational modifications, including acetylation, methylation, phosphorylation and ubiquitination [2]. Such covalent histone modifications generate another level of genetic regulation by influencing the state of chromatin. It is proposed that different combinations of modifications form a 'histone code' that permits the assembly of different epigenetic states, be it gene activation or gene silencing, and this leads to distinct outputs of genetic information [3–5]. Active centromeres in metazoans have yet another distinct form of chromatin in which the histone H3 variant CENP-A (centromere protein A) replaces canonical histone H3, and this is surrounded by blocks of 'heterochromatin'. A recent study set out to investigate whether there are additional defining chromatin marks in centromeric chromatin [6]. The authors introduce the term 'centrochromatin' to describe the distinct histone-modification pattern at centromeres. In addition, they have determined the relative 3D organization of these different types of chromatin at centromeres.

Histone modification patterns define different chromatin states

Three forms of methylated lysine residues are found in histones – mono-, di- and tri-methylation – and each form can denote a distinct chromatin state. Acetylation of histone H3, histone H4 and methylation of histone H3 on Lys4 (H3K4Me) are euchromatic marks that are associated with gene activity [7–9]. H3K4 di-methylation ($H3K4Me_2$) and H3K4 tri-methylation ($H3K4Me_3$) are associated with potentially active and transcriptionally active sites of the genome, respectively. By contrast, methylation of histone H3 on Lys9 (H3K9Me) is associated with transcriptionally silent chromatin and heterochromatin [10,11]. It is known that methylation of histone H3 on Lys4 and Lys9 can recruit specific regulatory factors such as Iswi1 (a chromatin remodelling factor) [12] or HP1 proteins (heterochromatin protein 1; chromodomain proteins) [13,14] that promote gene expression or gene silencing, respectively.

Specifying centromere identity

The centromere is the specialized region of the chromosome upon which the kinetochore assembles. Via its interaction with the microtubules of the mitotic spindle, the kinetochore ensures equal segregation of chromosomes at cell division. Given its importance, it is surprising that there is no conservation of centromeric sequences between different organisms and the definition of centromeric identity has remained enigmatic. In most multicellular eukaryotes, however, centromeres typically encompass megabases of highly repetitive DNA sequences, much of which is packaged in heterochromatin. Evidence from a variety of organisms, including human, *Drosophila* and fission yeast, suggests that centromeres are regulated in an epigenetic manner [15,16]. For instance, in *Drosophila* and humans, it is known that kinetochores can assemble at ectopic sites. These neocentromeres lack normal centromeric DNA and are propagated at these novel positions [17–19]. Therefore, in addition to their role in chromosome segregation, centromeres must preserve the site of kinetochore assembly through each cell cycle.

Although centromeric DNA is not evolutionarily conserved, a defining feature of all active centromeres is the presence of a variant histone H3, known as CENP-A in human and CID in *Drosophila*, which replaces histone H3 in nucleosomes exclusively in the kinetochore domain ('centromeric chromatin') of centromeres [20–22]. CENP-A/CID is required for the association of other kinetochore proteins and is a strong candidate for a protein that specifies and propagates the site of kinetochore assembly.

Specific modifications define centrochromatin

Previous analyses of CENP-A/CID-containing chromatin at human and fly centromeres have demonstrated that blocks of CENP-A/CID and H3 nucleosomes are linearly interspersed on extended chromatin fibres, and polynucleosomal fragments contain both H3 nucleosomes and CENP-A/CID nucleosomes [22]. By contrast, staining of

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metaphase chromosomes indicated that CENP-A/CID and H3 are in separate juxtaposed blocks at centromeres with CENP-A/CID at the outer face of the chromosome, and that H3 is concentrated internally. To reconcile these two observations, it was proposed that centromeric chromatin is looped or coiled so that the interspersed CENP-A/CID is amalgamated into a single entity (see later) to form a cylinder that faces outwards for kinetochore assembly and interaction with spindle microtubules, and the H3 is in an adjacent cylinder. However, the nature of this H3 remained unknown: it seemed likely that it might just be 'heterochro-heterochromatic' and exhibit $H3K9Me_2$ or $H3K9Me_3$ modifications. Surprisingly, it turned out to have its own distinct, conserved modification pattern – centrochromatin (Figure 1).

Immunolocalization was performed with a panel of antibodies that recognized specific histone modifications associated with either silent or active chromatin. Using CENP-A/CID as an indicator of centromeric chromatin, Sullivan and Karpen built up a picture of chromatin organization at the centromere by combining high-resolution 3D microscopy of metaphase chromosomes with analysis of linear chromatin fibres [6] (Figure 2a,b). In this staining technique, chromatin fibres on glass slides are extended to ~50–100 times the length their normal interphase appearance, enabling greatly improved resolution. On chromosomes, the CENP-A/CID domain (Figure 2b) lacks H3 and, therefore, the heterochromatic markers $H3K9Me_2$ and $H3K9Me_3$. However, $H3K9Me_2$ decorates flanking pericentromeric sites on linear chromatin fibres and stains the regions between and adjacent to CENP-A/CID cylinders on metaphase chromosomes (Figure 2a,b). The $H3K9Me_3$ modification was detected in pericentric regions that were not directly adjacent to the CENP-A/CID domain, and also localized to sites on chromosome arms, presumably, detecting other heterochromatin. Acetylated H3 and H4 were not detected in the CENP-A/CID domain or the flanking pericentric heterochromatin but were distributed at various non-centromeric sites on the chromosome arms, indicating the presence of active chromatin. Unexpectedly, $H3K4Me_2$ – which is normally associated with

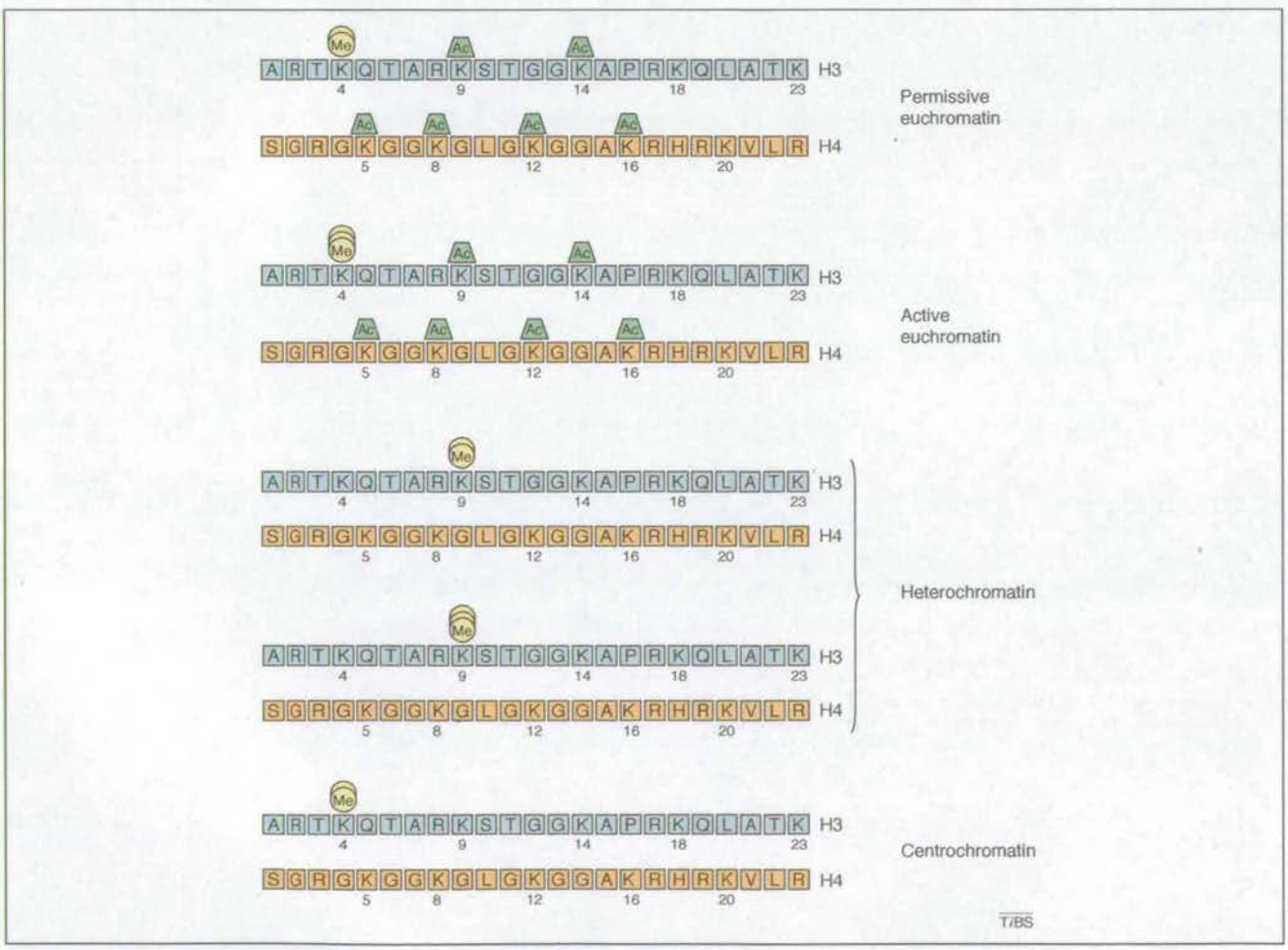

Figure 1. Centrochromatin bears a unique combination of histone-modification marks. The N-terminal tails of histones H3 (blue) and H4 (orange) are shown. Acetylation of lysine residues is indicated by green trapezoids. Modification by di-methylation and tri-methylation are indicated by two or three yellow circles, respectively. Only the modifications assessed in the paper by Sullivan and Karpen [6] are indicated, although many other modifications are known. Different chromatin states are marked by distinct combinations of post-translational modification. Centrochromatin contains histone H3 nucleosomes, which are di-methylated on Lys4 of histone H3 and hypoacetylated on other lysine residues in the H3 and H4 N termini and are interspersed with CENP-A nucleosomes in centromeric chromatin. Centrochromatin is, thus, distinct from both permissive and active euchromatin and from heterochromatin.

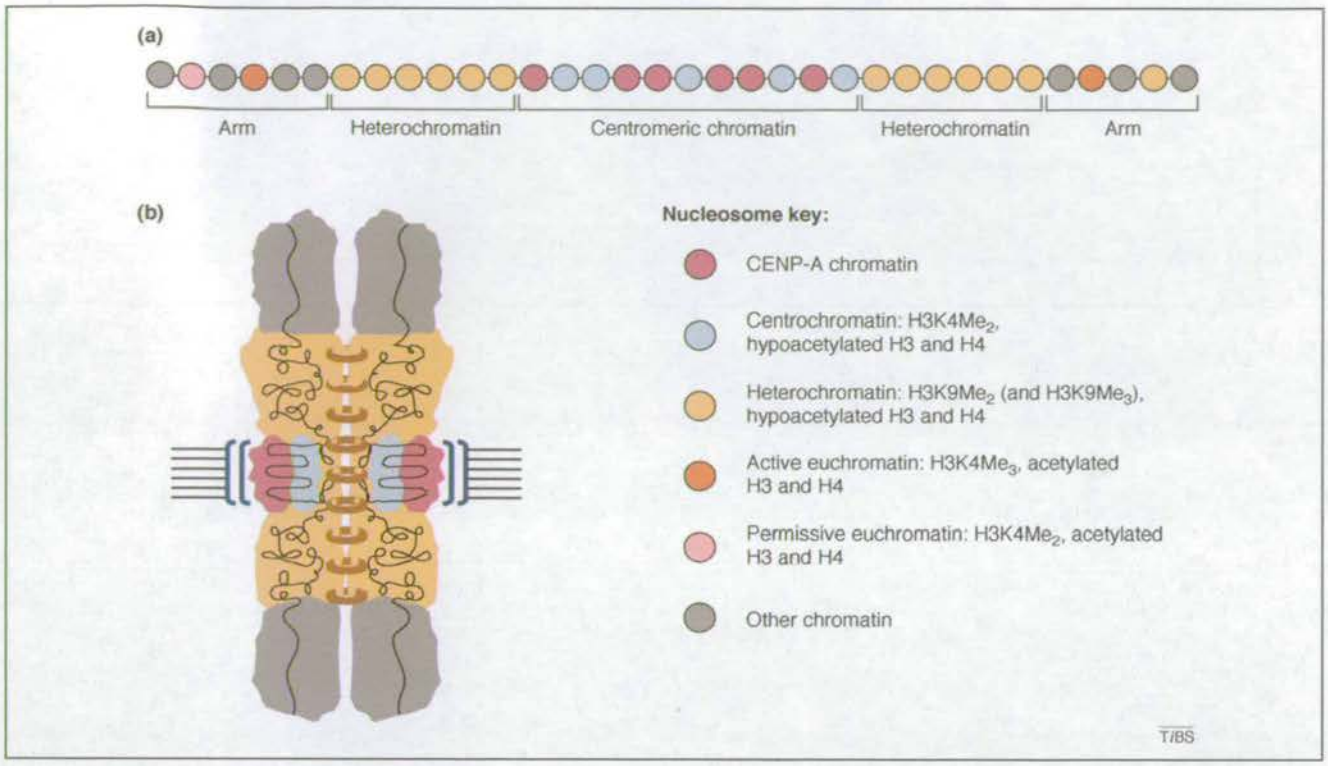

Figure 2. The structure and organization of centromeric chromatin. **(a)** Diagrammatic representation of an extended chromatin fibre. It represents a hypothetical chromosome based on the report by Sullivan and Karpen [6] and previous studies; not drawn to scale. The centromeric chromatin domain is marked by the presence of CENP-A-containing nucleosomes (purple), interspersed with H3-containing nucleosomes, which are uniquely marked by $H3K4Me_2$ and hypoacetylated H3 and H4 N-terminal tails (pale blue; 'centrochromatin'). Flanking the centromeric domain are blocks of heterochromatin (orange) that is marked by hypoacetylation of H3, H4 and $H3K9Me_2$ (and $H3K9Me_3$). The chromosome arms contain scattered sites of heterochromatin, active euchromatin (red), permissive euchromatin (pink) and other chromatin (grey). **(b)** 3D organization of centromeric chromatin in humans and *Drosophila*. The linear chromosome shown in (a) is organized into a 3D structure such that CENP-A/CID-containing nucleosomes are aligned in a cylindrical manner facing the poles of the mitotic spindle and forms the basis for the kinetochore (dark blue brackets; microtubules shown as thick black lines). Underlying the CENP-A/CID cylinder is a block of H3 nucleosomes marked by under-acetylation and di-methylation of Lys4 of histone H3 ('centrochromatin'). The black line shows the path of the chromatin fibre. The arrangement of the CENP-A/CID and domains in the centromeric chromatin could be achieved by looping (as shown) or coiling of the chromatin fibre. Underlying the centromeric domain and flanking it is the pericentric heterochromatin and, beyond that, the chromosome arms (grey). Cohesin is indicated by brown rings.

euchromatin – was found to be interspersed with the CENP-A/CID on extended fibres (Figure 2a). In addition, this $H3K4Me_2$ chromatin seems to underlie the CENP-A/CID-containing domain on metaphase chromosomes (Figure 2b). This demonstrates that centromeric chromatin contains histone H3 bearing a distinct histone modification, $H3K4Me_2$, which is usually found on chromatin that is poised and permissive for transcription. However, this chromatin – or centrochromatin – can be distinguished from active euchromatin by the absence of acetylated H3 and H4, a property normally associated with heterochromatin. By contrast, heterochromatin is methylated on Lys9 but lacks acetylation and H3K4 methylation (Figure 1).

Possible functions for centrochromatin

The study highlighted here clearly demonstrates that 'centrochromatin' represents a distinct form of modified chromatin that underlies CENP-A/CID chromatin at kinetochores. The function of this newly identified centromere-associated chromatin remains to be determined. It is possible that centrochromatin somehow serves to promote the assembly of CENP-A/CID nucleosomes on intervening nucleosomes. For example, it might function in concert with CENP-A/CID-specific chaperones or loading factors to orchestrate the safe delivery of CENP-A/CID to the centromere and, thus, contribute to the epigenetic inheritance of centromeric chromatin.

The 3D arrangement of centromeric chromatin also suggests the functional significance of these distinct domains: the CENP-A/CID nucleosomes amalgamate on the outer face of the chromosome, thus ensuring kinetochore assembly in a position ideal for interactions with spindle microtubules (Figure 2b). But how is the alignment of CENP-A/CID nucleosomes achieved? One possibility is that the $H3K4Me_2$ nucleosomes aggregate and, thus, arrange CENP-A/CID nucleosomes so that they are organized in a loop or coil above this anchor. The positioning of heterochromatin in flanking and intervening regions is ideally placed to perform functions such as sister-chromatin cohesion. It is also possible that this flanking heterochromatin contributes to the integrity of the centromeric chromatin by adopting an organizational role or by acting as a barrier to prevent the spreading or mixing of CENP-A/CID- and H3-containing chromatin.

Is the centromeric chromatin of other species marked and organized in a similar manner? The observed

conservation between human and *Drosophila* centrochromatin suggests that this might be the case, and it will be interesting to determine whether plant and mouse centromeres also contain blocks of H3K4Me$_2$ chromatin. Fission yeast centromeres consist of a central domain packaged in CENP-A nucleosomes flanked by twin blocks of heterochromatin, and it has been suggested that metazoan centromeres might represent iterations of this fission yeast centromere unit. Sullivan and Karpen [6] argue that their current results indicate that this is unlikely to be the case. The fission yeast central domain contains hypoacetylated H4 [23], but how much H3 it contains and whether it is in the form of H3K4Me$_2$ is currently unclear. It is possible that this H3K4Me$_2$ centrochromatin has arisen out of a need to organize multiple blocks of CENP-A/CID chromatin in more complex centromeres.

Concluding remarks

Although there seems to be no absolute *cis*-acting sequence that defines the site of kinetochore assembly on centromeres, there are clearly specialized chromatin features that define centromeric chromatin – the presence of CENP-A/CID and, now, H3K4Me$_2$-bearing centrochromatin. It is not known if this centromeric H3K4Me$_2$ is required for centromere function. One way to address this would be to correlate the presence of this centrochromatin with centromere activity: is there a concentration of H3K4Me$_2$ at human neocentromeres? Is it lost from an inactivated centromere? The identity of the histone methyltransferase responsible for methylation of the centrochromatin H3 is also unknown. Disrupting the activity of such a methyltransferase would help determine if this modification contributes in any way to centromere function. Many questions concerning the factors that govern the assembly of a functional centromere remain unanswered, however 'centrochromatin' is certainly making its mark.

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